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Departamento de Química

Diogo João Portugal-Nunes

Produção de Bioetanol a partir de SSLs: *S. stipitis* Vs *S. cerevisiae*

Bioethanol production from SSLs: *S. stipitis* Vs *S. cerevisiae*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia – Especialidade Biotecnologia Industrial e Ambiental (2º Ciclo), realizada sob a orientação científica da Doutora Marie Gorwa-Grauslund, Professora na Divisão de Microbiologia Aplicada da Universidade de Lund, Suécia, e da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, Portugal.

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Dedico este trabalho à minha família e amigos por terem sido vitais durante todo o meu percurso académico.

O júri

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Palavras-chave

Scheffersomyces (Pichia) stipitis, *Saccharomyces cerevisiae*, Licores de Cozimento ao Sulfito Ácido (SSLs), Imobilização celular, Matriz de alginato-cálcio; Bioetanol de 2ª geração

Resumo

O objectivo desta dissertação foi a comparação da produção de bioetanol de 2ª geração por *Scheffersomyces stipitis* (em suspensão e imobilizada) e por duas estirpes industriais de *Saccharomyces cerevisiae*. Os substratos utilizados para a realização das fermentações foram os Licores de Cozimento ao Sulfito Ácido (SSLs) provenientes de madeira de resinosas (HSSL) e de madeiras de folhosas (SSSL – Domsjö hydrolysate). O HSSL e o SSSL são sub-produtos da indústria de pastas de papel, resultando do processo de cozimento ao sulfito com magnésio e sódio, respetivamente. Além de lenhosulfonatos, estes SSLs contêm monossacarídeos, destacando-se a glucose, xilose e manose. O ácido acético, composto inibidor da fermentação alcoólica por leveduras, também está presente em concentrações relativamente elevadas (≥ 5.0 g . L^{-1}). O HSSL foi pré-tratado físico-quimicamente e submetido a uma remoção biológica de inibidores com *P. variotii*, enquanto o Domsjö hydrolysate foi utilizado sem qualquer tratamento. *S. stipitis* e *S. cerevisiae* são leveduras extensamente estudadas devido à sua capacidade de fermentação de pentoses e hexoses, respetivamente.

Num estágio prévio às fermentações dos SSLs, as leveduras foram pré-adaptadas em 60% HSSL ou 40% SSSL. Os máximos de rendimento (0.440 g etanol . g açúcares^{-1}) e produtividade em etanol (0.885 g etanol . L^{-1} . h^{-1}) foram obtidos nas fermentações com *S. cerevisiae* TMB 3500 em SSSL, sendo estas as variáveis com maior potencial para aplicação industrial. Embora a cultura suspensa de *S. stipitis* tenha resultado numa menor produtividade (0.010 g etanol . L^{-1} . h^{-1}), a optimização do fornecimento de oxigénio ao biorreator deverá conduzir ao aumento da produtividade volumétrica em etanol.

A imobilização celular e o controlo do pH em 5.5 nas fermentações com *S. stipitis* melhoraram a eficiência fermentativa ao aumentarem a produção de etanol em 1.3 e 1.6 vezes, respetivamente. Quando aplicadas simultaneamente, estas duas condições aumentaram o rendimento em etanol 2.2 vezes, sugerindo que (i) a imobilização numa matriz de alginato de cálcio protegeu a levedura dos inibidores químicos e que (ii) o controlo de pH em 5.5 foi determinante para a produção de etanol a partir de HSSL biologicamente pré-tratado.

Os resultados comprovam que ambos os SSLs são potenciais substratos para a produção de bioetanol de 2ª geração usando *S. stipitis* ou *S. cerevisiae*, sob o conceito de biorefinaria.

Keywords

Scheffersomyces (Pichia) stipitis, *Saccharomyces cerevisiae*, Spent Sulphite Liquors (SSLs), Cell immobilization, Calcium-alginate matrix, Second-generation bioethanol

Abstract

The aim of this work was the comparison of second-generation bioethanol production by *Scheffersomyces stipitis* (free-culture and immobilized) and two *Saccharomyces cerevisiae* industrial strains, using Hardwood Spent Sulphite Liquor (HSSL) or Domsjö hydrolysate (SSSL) as substrate. HSSL and SSSL are side products of pulp and paper industry, from magnesium and sodium-based acidic sulphite pulping, respectively. Besides sulphonated lignin, SSLs contain fermentable sugars, mainly glucose, xylose and mannose. Acetic acid, a known inhibitor of ethanol fermentation by yeasts, is also present in a relatively high content (≥ 5.0 g . L^{-1}). HSSL was previously physico-chemically pretreated and bio-detoxified with *P. variotii*, whereas SSSL was used without any treatment. *S. stipitis* and *S. cerevisiae* are the most widely studied pentose and hexose-fermenting yeasts, respectively.

Before fermentations in SSLs, all yeast strains were pre-adapted by growing them in 60% HSSL or 40% SSSL. The highest maximum ethanol yield (0.440 g ethanol . g $sugars^{-1}$) and productivity (0.885 g ethanol . L^{-1} . h^{-1}) were obtained using *S. cerevisiae* TMB 3500 and SSSL. Suspended S. stipitis achieved a lower ethanol productivity (0.010 g ethanol . L^{-1} . h^{-1}) but further optimization on the supplied oxygen in the fermentor might lead to higher ethanol productivity. Immobilization and pH control at 5.5 on *S. stipitis* fermentations improved the fermentation efficiency, increasing up the ethanol production by 1.3-fold and 1.6-fold, respectively. When applied simultaneously, these two conditions increased the ethanol yield 2.2-fold, suggesting that (i) immobilization with a calcium-alginate matrix protected the yeast from the inhibitory compounds and (ii) the controlled pH at 5.5 was essential for ethanol production from bio-detoxified HSSL.

Results showed that both SSLs are potential substrates for the production of 2nd generation bioethanol by *S. stipitis* or *S. cerevisiae*, under the biorefinery concept.

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Abbreviations and symbols index

CFUs - Colony-forming unit

Effic. - Fermentation efficiency considering the maximum theoretical ethanol yield (0.51 . g ethanol . (g sugars)$^{-1}$)

Furfural - 2-furaldehyde

GHG - Greenhouse gas emission

GRFA - Global Renewable Fuels Alliance

HMF - 5-hydroxymethyl-2-furaldehyde

HSSL - Hardwood Spent Sulphite Liquor

LCB - Lignocellulosic biomass

NA - Not applicable

P_E 20-25h - Ethanol productivity after 20-25h of fermentation (g ethanol . (L . h)$^{-1}$)

P_E Ymax - Volumetric ethanol productivity at the maximum yield (maximum g ethanol . (L . h)$^{-1}$)

P_E - Volumetric ethanol productivity (g ethanol . (L . h)$^{-1}$)

P_0 - Weight (grams) at the beggining of the Wet weight evaluation

P_f - Weight (grams) at the end of the Wet weight evaluation

P.O. - Propylene oxide

PPP - Pentose-phosphate Pathway

r_S Ymax - Substrate (glc + xyl + acetate) consumption rate at the maximum ethanol yield (g substrate . (L . h)$^{-1}$)

SSLs - Spent Sulphite Liquors

SSSL - Softwood Spent Sulphite Liquor

TMB 3500 - *Saccharomyces cerevisiae* TMB 3500

TMB 3720 - *Saccharomyces cerevisiae* TMB 3720

XDH - Xylitol dehydrogenase

XI - Xylose isomerase

XR - Xylose reductase

wt - *wild-type*

$Y_{P/S}$ max - Maximum ethanol yield (maximum g ethanol . (g glc + g xyl)$^{-1}$)

YNB - Yeast Nitrogen Base

YM - Yeast Medium

YPD - Yeast Extract Peptone Dextrose

[P] - grams of Wet Weight per mL of SSL

μ_{max} - Maximum specific growth rate

***** - Data not available

1. Introduction

1.1. Lignocellulosic biomass

Lignocellulosic biomass (LCB) is the most abundant renewable carbohydrate resource on Earth, comprising about 50% of world biomass [1, 2], and it can be classified in four categories according to its origin:

- Wood;
- Non-food agricultural crops and residues;
- Municipal solid wastes;
- By-products and/or wastes from pulp and paper industries [3-5].

The composition of LCB depends on the plant species and consists primarily of cellulose, hemicelluloses and lignin, which are the integral part of cell wall in plant tissues and are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages [6]. Cellulose and hemicelluloses are hydrolysable structural polymers of cell wall and the main sources of fermentable sugars [3, 7]. Cellulose is the most abundant structural polysaccharide (30-55% abundance in the cell wall), comprised by repeated β-D-glucopyranose units linked by β(1→4)-glycosidic bonds [1]. However, its amorphous-crystalline structures leads to recalcitrance and that makes it poorly accessible and resistant to hydrolysis [8]. Hemicellulose, which contributes to 10-40% of plant material, is composed of heteropolysaccharides chemically linked to lignin in the cell wall. It is constituted by pentoses, mainly D-xylose and L-arabinose, and hexoses, mainly D-mannose, D-galactose and D-glucose [1, 8]. The structure and the composition of hemicelluloses vary significantly among plant species and the most abundant are xylans followed by mannans and galactans [1]. Lignin, which contributes to 15-30% of plant biomass, can be used as an ash-free solid fuel for heat and/or energy production and for the production of advanced materials, polymers and aromatic aldehydes [1, 3, 9-11].

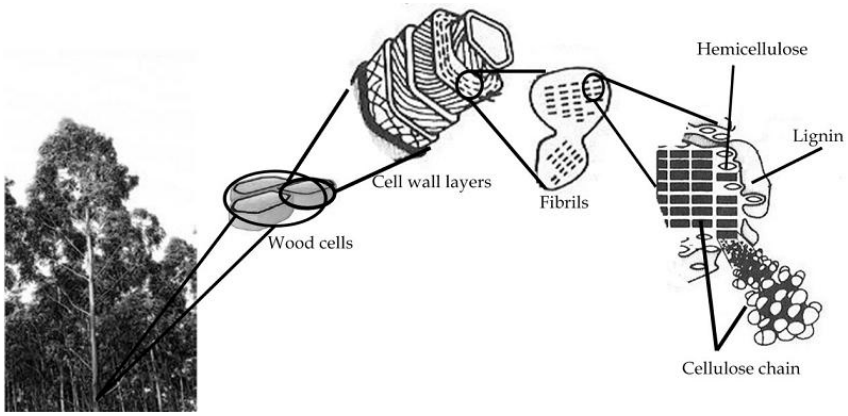


Figure 1 – Schematic representation of wood plant cell wall and its macromolecular components [1].

Nowadays, LCB are widely used in unmodified state as e.g. drilling muds, cement and concrete additives, emulsifiers/stabilizers, grinding aids, binders/adhesives, resin ingredients, rubber additives or tanning agents [12]. In addition to these interesting properties and applications, the importance of LCB and its derivatives as feedstock for bioethanol production and for the development of other bio-based products has been growing [1]. This raw material is less expensive than conventional agricultural feedstock and can be produced with lower input of fertilizers, pesticides, and energy; it is outside of the human food chain, allowing biofuel production without using arable land, and its energetic content largely exceeds the world basic energy requirements [3, 9, 13]. However, it is crucial to optimize the conversion of lignocellulose into fermentable sugars and further to ethanol. The heterogeneity of the feedstock and the influence of specific process conditions on microorganisms and enzymes performance must be overcome in order to attract the industrial interest and investment for research and development of second-generation biofuels [11, 13, 14].

1.2. Wood

At a fundamental level, wood is a complex biological structure, itself a composite of many chemistries and cell types acting together to serve the needs of the plant [15]. This renewable resource is mainly composed by three natural polymers: cellulose, hemicelluloses and lignin, existing also residual amounts of extractives and ashes [15, 16]. These polymer substances are not uniformly distributed within the wood cell wall and their concentrations change from one morphological region to another [16]. This fact leads to the existence of different types of wood, which are commonly classified as softwoods (gymnosperms) and hardwoods (angiosperms), and both are widely distributed on earth, from tropical to arctic regions [16].

Wood is recyclable, renewable, and biodegradable and many species are shock resistant and, bendable [15]. Besides that, it can be converted into many useful industrial chemicals and products, such as ethanol, plastics and paper [15].

Because of its unique properties, wood has remained an important material throughout history and has a large economic impact in several countries in the world [15, 16].

• 1.1.1. Softwood

Softwoods are the dominant source of lignocellulosic materials in the Northern hemisphere and during recent years have been subject of great interest in Sweden, Canada and USA as a renewable source for ethanol production [17-19]. This type of wood contains approximately 43-45% cellulose, 20-23% hemicellulose and 25-35% lignin [11]. Hemicelluloses are mainly

constituted by mannose, which is an hexose that can be fermented by *Saccharomyces cerevisiae*, and the content of pentoses is only around 6-7% of the total wood [11].

- **1.1.2. Hardwood**

The anatomy of hardwoods is more complex and less studied than softwoods, but during recent years there is an increasing interest in this type of wood. Hardwood cells are mainly composed by fiber, vessel and parenchyma, and the physical and chemical properties are closely related to the presence of fibers [16].

Cellulose comprises between 40-55% of total hardwood, followed by hemicelluloses (20-35%) and lignin (18-25%), which usually is less than in the softwoods [16]. Hemicelluloses are defined as amorphous heterogeneous polymers, being xylans and glucomannans the two predominant types in hardwoods [20, 21]. The percentage of these two components varies with the tree species, but generally it contains a higher amount of xylans (15-30%) and only 2-5% of glucomannans [8].

1.3. Sulphite Spent Liquors

Pulps are the basic and most important material obtained from the wood processing, being used for papermaking, cellulose derivatives, chemical compounds, and plastics [8]. The wood pulping processes to obtain cellulose fibers (pulps) from wood can be done chemical and/or mechanically among others [8, 15, 22]. The dissolution of wood components during pulping varies according to the pulping process employed [23]. Chemical wood pulping is the most common in industries. In addition to the lignin removal, with chemical compounds conjugated with low pH (1.5 - 2.0) and high temperatures (130-170^{o}C), some degradation of the monomeric sugars sources from cellulose and hemicellulose occurs [8, 15]. The cooking process time period has to be chosen according to the balance between the higher delignification and the preservation of cellulose fibers integrity enough to allow satisfactory pulps production yields.

Spent sulphite liquors (SSLs) are the side product from acidic sulphite or bisulphite wood pulping processes and their major components are lignosulphonates, which are usually burned for energy and chemicals regeneration [8, 24]. In this wood cooking process the acid is composed by sulfur dioxide (SO_2) and a cation linked to the bisulfite ion (HSO_3^-). Magnesium is the dominating cation used in sulphite pulping, generating $Mg(HSO_3)_2$, however calcium (Ca^{2+}), sodium (Na^+) or ammonium (NH_4^+) can also be used [8, 15]. Usually, the cooking process is done during approximately 8 hours at 135^{o}C but these conditions may vary slightly depending on the processing company (Figure 2). The wood cooking results in unbleached pulp, which has to be submitted to a

bleaching process in order to remove lignin residues in the pulp. After that a washing step is needed to further use this pulp for papermaking and chemical feedstock production [1]. The residue of this process is evaporated and burned in a boiler in order to allow the regeneration and re-use of the cooking chemicals. The SSLs can be obtained right before or after evaporation (Figure 2) [1, 25]. The wood cooking process uses relative hard conditions of temperature and pH, which leads to the formation of chemical compounds that are microbial inhibitors.

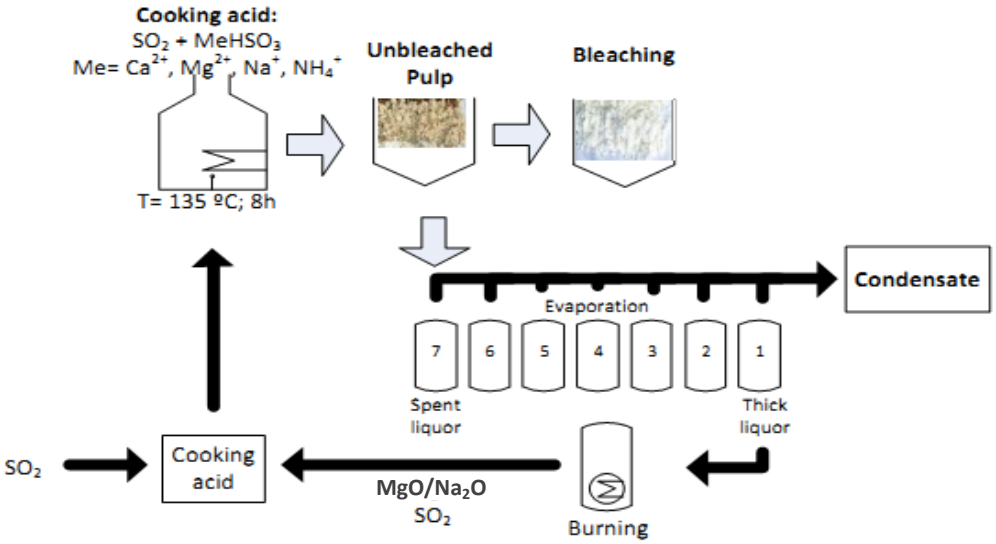


Figure 2 – Acidic sulphite wood cooking process with production of SSL [1] .

The chemical composition of SSLs depends on the wood species used for the pulping due to the differences in molecular weight, structural features of lignosulphonates and saccharide composition. This information is essential regarding the eventual SSLs utilization for different purposes and commercial applications, such as biofuels, polymers or protein production [8, 23, 26, 27]. The main characteristics of two examples of different SSLs are described in the following sections:

- Softwoods (SSSLs – Softwood Spent Sulphite Liquors): Domsjö Hydrolysate;
- Hardwoods (HSSLs - Hardwood Spent Sulphite Liquors): Caima HSSL.

- **1.3.1. Domsjö Hydrolysate**

Domsjö hydrolysate is produced at Domsjö Fabriker AB in Sweden and is an example of a SSSL. This liquor is a disodium-based wood cooking process obtained from a controlled mixture of spruce (*Picea abis*) and Pine (*Pinus sylvestris*). Domsjö hydrolysate is collected after some evaporation steps - represented in Figure 2 as *thick liquor*.

The organic matter of SSLs obtained from acidic sulphite pulping of softwood (SSSLs) consists essentially of water-soluble lignosulphonates (sulphonated lignin) and hexoses arisen from

the hydrolysis of glucomannan [8]. Domsjö SSSL contains a mixture of pentose and hexose sugars which indicates that this liquor constitutes a good feedstock for bioethanol production, since *S. cerevisiae* and *Zymomonas mobilis* are able to efficiently ferment hexoses into ethanol [28-31]. This SSSL also contains inhibitory compounds that are generated during the acid sulphite cooking, and notably acetic acid, glucuronic acid, 5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (furfural) and phenolic compounds (Table 1) that may affect yeast fermentation. This topic is further developed in section 1.3.3.

Table 1 – Composition of the SSSL inhibitors from Domsjö Fabriker [28].

Compound	Concentration (g . L^{-1})
Acetic Acid	5.3
Glucuronic Acid	0.2
5-hydroxymethyl-2-furaldehyde (HMF)	0.5 ± 0.1
2-furaldehyde (Furfural)	0.2 ± 0.1
Phenolics	20 mM

- **1.3.2. CAIMA HSSL**

HSSLs as feedstock for the production of ethanol and other value-added compounds are less widely studied than SSSLs. Only scarce knowledge about the chemical structure is available, especially for the lignosulphonates from magnesium-based acidic sulphite pulping of *Eucalyptus globulus* wood [1, 23, 32].

Caima HSSL is produced at CAIMA, S. A. industry, in Portugal, and it is a result of a magnesium-based *Eucalyptus globulus* wood cooking collected from the first evaporator - represented in Figure 2 as *spent liquor*. This specific liquor contains lignosulphonates and hemicellulose hydrolysis products, mainly pentoses from the glucuronoxylan fraction. D-xylose corresponds to more than 50% of total sugars (Table 2), fact that makes this specific HSSL a suitable substrate for highly efficient pentose-fermenting yeasts such as *Scheffersomyces stipitis*, *Candida shehatae* and *Pachysolen tannophilus* [27, 29, 32, 33]. However, there are high amounts of acetic acid (9.6 g . L^{-1}) and traces of furfural, HMF, polyphenols and low molecular weight lignosulphonates like gallic acid, pyrogallol and syringic acid (68.0, 6.3 and 3.6 mg per 100g of HSSL, respectively) that act as inhibitors of the microbial metabolism. This is the main drawback for HSSL bioprocessing [32, 34]. The classification and the effect of these inhibitory compounds are explained in the following section.

Table 2 - Composition of the HSSL from CAIMA, SA [27, 35] .

Compound	Concentration (g . L^{-1})
Sugars	44.7 ± 0.0
- D-xylose	24.6 ± 0.5
- D-mannose	8.5 ± 0.9
- L-arabinose	7.8 ± 0.3
- D-galactose	4.5 ± 0.1
- D-glucose	2.3 ± 0.1
- L-ramnose	1.6 ± 0.3
- L-fucose	0.4 ± 0.3
Acetic Acid	9.6 ± 0.1
5-hydroxymethyl-2-furaldehyde (HMF)	Traces
2-furaldehyde (Furfural)	Traces
Lignosulphonates	78.2 ± 0.6
Dry solids	150 ± 0.8

- **1.3.3. Inhibitors**

Microbial inhibitors may drastically affect cell growth, substrate uptake and/or other related metabolic pathways [34, 36, 37]. *Mussato et al. (2004)* referred that the maximum concentration of inhibitor that can be used without significant effects in the media is dependent on the compound itself, the microorganism used in the process and its physiological state, the fermentative process technology, the dissolved oxygen concentration in the medium and the pH [34].

According to the origin, inhibitors can be classified in four groups: sugar degradation products, lignin degradation products, compounds derived from extractives and heavy metal ions [34, 37].

HMF and furfural are furan derivatives formed by dehydration of hexoses and pentoses, respectively, and their levels depend on the type of raw material and the pretreatment [38]. HMF and furfural decrease the volumetric ethanol yield and productivity. They inhibit cell growth, decreasing the specific growth rate and cell-mass yield and/or extend the lag phase [37, 39, 40]. These effects depend on the furans concentration and yeast strain used, but in most of the studies HMF has a less significant negative impact than furfural on fermentations.

Taherzadeh et al. (2000) reported the existence of a synergistic effect of HMF and furfural, and *Mussato et al.* (2004) found an increase of these inhibitory effects when furans were combined with other lignin degradation products [34, 41]. *S. cerevisiae* strains are able to slowly convert HMF and furfural to their respective alcohol forms. Still significant negative effects were found in the specific ethanol productivity rate with 2.0 g . L^{-1} HMF or 0.8 g . L^{-1} furfural [39, 42]. Using 2.0

g . L^{-1} of both furans simultaneously, a growth inhibition of 72% and a delay lag phase of more than 24 hours were detected [43]. Other investigations showed that *S. stipitis* is not affected by 0.5 g . L^{-1} furfural [44]. However the presence of 1.5 g . L^{-1} furan derivative interfered in respiration and cell growth, decreasing 90% the ethanol yield and 85 % the productivity [33].

Acetic, formic and levulinic acids are examples of the weak acids that can be found in SSLs and are formed during hemicellulose hydrolysis [42]. Acetic acid (acetate) is a known microbial inhibitor that is formed by de-acetylation of hemicelluloses and is present in high concentrations (8-9 g . L^{-1}) in SSLs [1, 32, 45]. Oxygen content, pH medium and acetic acid concentration influence the degree of inhibition of this organic acid [45]. In addition, HMF breakdown leads to the formation of formic and levulinic acids. Formic acid can also be generated from furfural under acidic conditions at elevated temperatures [38, 39]. These week acids inhibit microorganisms, affecting negatively the specific growth rate, ethanol yield and productivity. *Larsson et. al* (1999) concluded that there were not synergistic effects among these three acids [46]. In undissociated form at low pH medium, weak acids can diffuse across the cell membrane leading to the decrease of cytosolic pH [7, 34, 47, 48]. The decrease in intracellular pH is compensated by the activity of the plasma membrane ATPase and less ATP is available for biomass formation, causing cell activity inhibition or cell death [1, 48]. Besides, *Bauer et al.* (2003) showed that week organic acids also reduce the uptake of aromatic amino acids from the media, inhibiting yeast growth [49]. However, some studies found that low levels of acetic acid, levulinic acid or formic acid, individually, may increase the ethanol yield [42, 50].

During sulphite acid wood cooking process, aromatic, polyaromatic, phenolic, and aldehydic compounds can be generated from lignin degradation [23]. These inhibitory compounds affect the integrity of biological membranes, causing an inefficient sugar uptake and, consequently, a decrease in the maximum specific growth rate [37, 51]. All the referred residues are more inhibitory to yeasts than HMF and furfural, and the phenolic compounds are the most toxic [36, 52, 53]. Gallic acid and pyrogallol are low molecular weight phenolics with antifungal properties [54, 55].

The extractives are less toxic to microbial growth than lignin derivatives [34]. The heavy metals that result from equipment corrosion during basic or acidic pulping processes can also be toxic for microorganisms.

Almeida et al. (2007) found that there is a synergistic effect when the furan derivatives, weak acids (mainly acetic acid) and phenolic compounds are present simultaneously [39].

- 1.3.3.1. SSLs detoxification

The toxic compounds formed during wood cooking may significantly affect microbial growth and fermentative alcoholic metabolisms [34, 56, 57]. Thus, their presence is the main drawback for bioprocessing SSLs since they affect fermentation efficiency and ethanol productivity [32, 34]. Hence, the removal of inhibitors, yeast strain adaptation and/or genetic/metabolic engineering are crucial steps to achieve economic feasible bioprocesses [37, 58, 59].

In order to detoxify the SSLs and other lignocellulosic by-products, physico-chemical and biological methods have been applied for the specific removal of inhibitors prior to fermentation [37]. Physico-chemical methods involve extraction techniques with specific solvents and chemical detoxification can be achieved by alkali treatment with calcium hydroxide ($Ca(OH)_2$) and sodium hydroxide (NaOH) [37]. One example of a biological approach is the use of peroxidase and laccase from the fungus *Trametes versicolor*, which increases the maximum ethanol productivity, since the enzymes are responsible for complete removal of phenolic compounds [60]. *Palmqvist et. al* (2009) showed that *Trichoderma reesei* is also able to degrade inhibitors in lignocellulosic substrate, leading to better ethanol yields [61]. More recently, *Pereira et. al* (2011) found that the biological detoxification of eucalyptus HSSL with *Paecilomyces variotti* is an efficient way to reduce the content of acetic acid and low molecular weight phenolics, allowing a further productive ethanol fermentation with another microorganism [27].

The removal of inhibitors from SSLs is a difficult task because the content varies with the wood type and wood cooking process conditions, and the detoxification step should not degrade sugars. It is also important to refer that inhibition degree vary with the strain in use. Hence, detoxification processes must be chosen according to all these factors, in order to achieve a sustainable and economic feasible process and large-scale production [27, 37, 53].

1.4. Biofuels

During recent years, interest in finding alternative energy sources for fossil fuels has been increasing due to the depletion of oil resources (petroleum, natural gas and charcoal), to the associated negative environmental impact (greenhouse gas emission (GHG) and global warming) and to the progressive grow on world energy requirements [32, 62]. Since world reserves of fossil fuels are limited and close to finish, many countries are actively researching for non-petroleum, renewable and non-polluting fuels – biofuels - to face the needs of energy supply and to response to climate change issues [32, 63]. European Union and other international committees have created new directives to incentive and oblige member-countries to research biotechnological solutions for

energy and chemical demands from renewable resources. The EU directive 2009/28/CE states that 10% of traffic fuels shall be biofuels by 2020 [32].

Biofuels are renewable, simple to use, biodegradable, nontoxic, essentially free of sulfur and aromatics, can be mixed with any proportion of petroleum to create a biofuel blend and, at last, they can be used in conventional heating equipment or diesel engines without major modification [62]. They may be produced from domestic biomass, by-products from industrial processing or from the recovery and reprocessing of products such as cooking, vegetable oil and other residues.

Table 3 – Classification of biofuels based on their production technologies, adapted from *Demirbas* (2009) [64].

Generation	Feedstock	Examples
First Generation Biofuels	Sugar, starch, vegetable oils or animal fats	Bioalcohols, vegetable oil, biodiesel, biosyngas, biogas
Second Generation Biofuels	Non-food crops, wheat straw, corn, wood, solid waste, energy crops, industrial by-products/wastes	Bioalcohols, bio-oil, bio-DMF, biohydrogen, wood-diesel
Third Generation Biofuels	Algae	Vegetable oil, biodiesel
Fourth Generation Biofuels	Vegetable oil, biodiesel	Biogasoline

Depending on the origin of the raw-material and the used technology, biofuels can be classified in four sections or generations (Table 3). First generation biofuels are controversial because of the competition with food production for arable lands. Thus, research is now more being focused on investigation and optimization of second-generation biofuels, that do not compete with the food chain and reduce the accumulation of residues by bioprocessing [1, 5, 32]. A large number of research studies have demonstrated the potential of biomass to efficiently replace oil in a long term, by producing second or further generation fuels, power, chemicals and/or materials feedstock [3, 62, 65-69]. However, is still necessary to optimize processes and technologies in order to reduce biofuels production costs and compete with fossil fuel based processes prices.

Biogas, biomethanol, biodimethylether, biohydrogen and pure vegetable oils are all claimed as biofuels, but bioethanol and biodiesel are the most widely recognized sources for the transport sector. These two biofuels, by themselves or as blends, can help to cut substantially oil imports and carbon emissions [62, 70]. Figure 3, from 2008, shows and previews the evolution of bioethanol and biodiesel production worldwide [71].

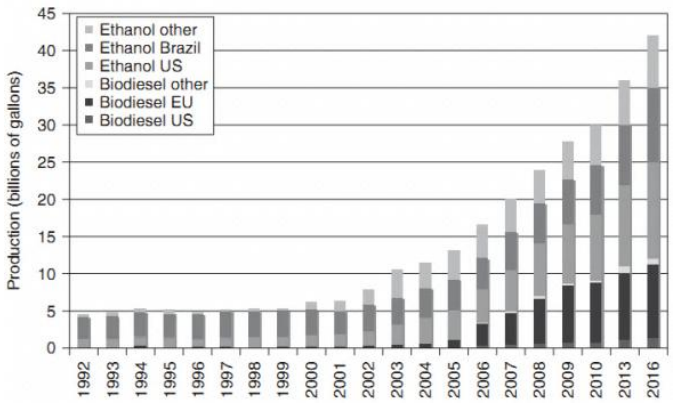


Figure 3 - World biodiesel and ethanol historical production and projections [71].
US – United States of America; EU - Europe

Biofuels constitute a viable energy source for the foreseeable future, replacing, at least partially, the fossil fuels. Thus, bio-renewable fuels seem to be promising and to have the potential to become the new base for socioeconomic development and environmental concerns [62].

- **1.4.1. Bioethanol**

Bioethanol, the most common biofuel, is a widely studied alcohol that can be produced by direct fermentation of sugars from several raw materials such as agricultural products or industrial wastes [65, 67]. The high octane number and high heat of vaporization are examples of the advantages of using bioethanol has a transportation fuel [72, 73].

Balat et al. (2008) classified bioethanol feedstocks into three types, that are commonly used [4]:

- lignocellulosic materials (woody biomass, herbaceous perennials, SSLs and various wastes);
- starch-rich crops (maize and grain sorghum);
- sucrose-rich crops (sugarcane and sugar beet).

Lignocellulosic biomass and its derivatives are more complex substrates to convert to bioethanol than simple sugars or starch. However, the non-interference with food chain and production, and the possibility of non-using arable lands compensate its complex drawback [1, 3, 9]. Besides, this raw material is less expensive than conventional agricultural feedstock and can be produced with lower input of fertilizers, pesticides, and energy. Ethanol from lignocellulosics generates low net GHG emissions, reducing environmental impact [1, 5, 74].

First-generation bioethanol production technology is well established in some countries but new methodologies have been developed in the last years in order to achieve new and non-cost intensive methods and processes for bioconversion of lignocellulosic renewable resources (e.g. SSLs) [62].

A

Country	Production (Ml/year)	
United States	40 130	54%
Brazil	24 900	34%
China	2 050	3%
Canada	1 348	2%
France	1 250	2%
Germany	750	1%
Spain	465	1%
Thailand	401	1%
India	350	-
Colombia	310	-
Australia	220	-
Austria	180	-
Sweden	175	-
Poland	166	-
Hungary	150	-
Others	1 110	2%
TOTAL	73 954	100%
- of which EU	3 703	5%
- of which Switzerland	0	-

B

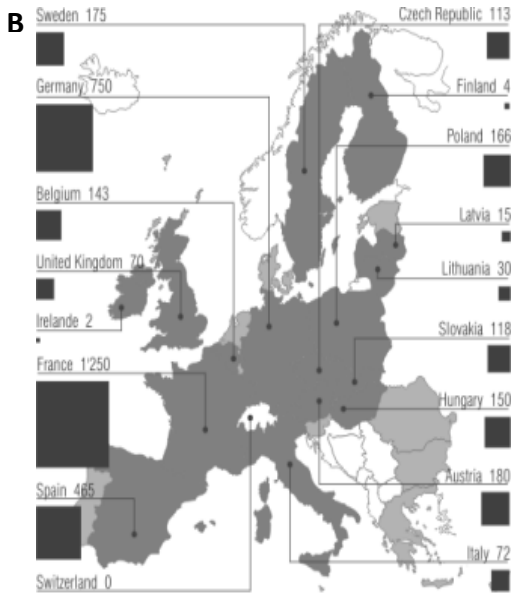


Figure 4 - World bioethanol production in 2009, in Million liters (Ml) per year (4-A) [75]. Production of bioethanol in the EU countries and Switzerland in Ml per year (4-B) [76].

In 2009, global production of bioethanol reached 75 billion liters (Figure 4), which was more than 94% of global biofuel production, and the preliminary 2012 report of *Global Renewable Fuels Alliance (GRFA)* indicates that the production is now about 85 billion liters per year [75-77]. According to the projections, global production should increase less in the next years and then reach a stable level [74, 78, 79]. Brazil and the US represent the world leaders, together accounting for about 90% of the world bioethanol production [75, 79]. However, these two countries produce only 1st generation bioethanol from their large production of sugarcane (Brazil) and crops, mainly maize, (US), which ends in large amounts of sugars and starch, respectively [80, 81]. In Figure 4, it is possible to conclude that in 2009, the bioethanol production by the European Union countries was very low, representing only 5% of the world production. From 2009 to 2012, the EU production increased almost 2 billion liters. However the percentage of world production remains about 5% [75, 76].

- 1.4.1.1. Bioethanol production from lignocellulosic feedstock

Ethanol can be produced from lignocellulosic material in several ways, but all processes involve common steps (Figure 5): pretreatment of raw materials; hydrolysis of polysaccharides (cellulose and hemicellulose) into monomer sugars; fermentation of monomer sugars and, finally, product recovery and concentration by distillation [4, 11, 30]. Usually, the main difference between the process alternatives is the hydrolysis step that can be performed by dilute acid, concentrated acid, dilute base or enzymatically [11, 82].

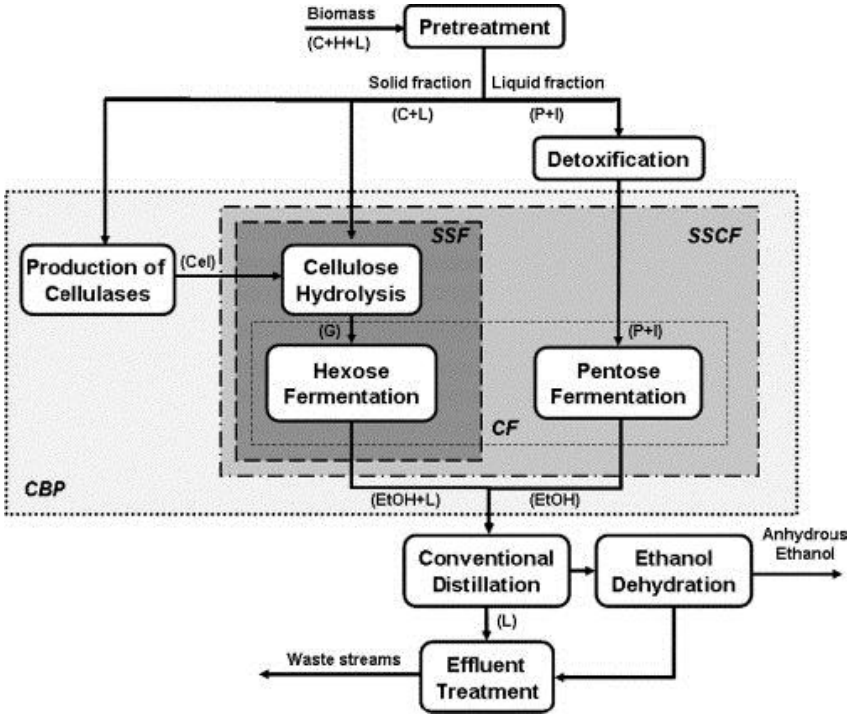


Figure 5 – Block diagram with examples of ethanol production processes from lignocellulosic biomass. Possibilities for reaction–reaction integration are shown inside the shaded boxes: CF (co-fermentation), SSF (simultaneous saccharification and fermentation), SSCF (simultaneous saccharification and co-fermentation) CBP (consolidated bioprocessing). Main stream components: C - Cellulose; H - Hemicellulose; L - Lignin; Cel - Cellulases; G - Glucose; P - pentoses; I - inhibitors; EtOH – ethanol [83].

Since bioethanol production from lignocellulosic material started being developed, a wide variety of microorganisms has been tested, including fungi, bacteria and yeasts. Bioethanol is mostly produced by fermentation of monomeric sugars by mesophiles, at 25-37ºC. However, some thermophiles have been tested and have some associated advantages [84]. In 2007, *Hahn-Hägerdal et al.* stated four basic requirements to a microorganism be considered as a potential bioethanol producer: process water economy, inhibitor tolerance, ethanol yield and specific ethanol productivity [53].

Process water economy means that the bioprocess should be planned to consume the minimum water content possible in order to reduce the added costs to the final product [53]. The thermophilic microorganisms represent an advantage in this specific field, since there is no need of cooling processes with fresh water. Besides, with this specific kind of microorganisms, the contamination risk is much lower because only thermophilic organisms are able to grow [84, 85].

An efficient producer should also be tolerant to the chemical inhibitory compounds that are formed during LCB pretreatment, treatment and/or hydrolysis process. There are some detoxification methods that partially remove these inhibitors, but a microorganism should have a

high tolerance level in order to reduce the final cost. *S. cerevisiae* is a good example of a microorganism that is able to tolerate high amounts of chemical inhibitors, without reducing the fermentation efficiency [38, 59].

In an industrial context, a bioethanol producer must be robust, osmotolerant and inhibitor-tolerant and, specially, be an efficient ethanol producer from sugars present in lignocellulosic feedstocks. Since the raw materials cost contributes in about one third to the final product cost, it is essential to use a microorganism able to efficiently convert all sugars of the lignocellulosic feedstock. Besides, the sugars must be converted to ethanol with minimal by-product formation [38]. Volumetric and specific productivities are also two of the most important parameters to evaluate process efficiency. The ethanol volumetric productivity ($g \cdot L^{-1} \cdot h^{-1}$) is proportional to the amount of biomass used in the fermentation step and is related to investment costs. Generally, high cell density fermentation increases this parameter and reduces the investment cost. However, there must be a balance, since biomass production is a function of the added nutrients price. The efficiency of the fermentative microorganism can be measured by the specific ethanol productivity ($g\ ethanol \cdot g\ biomass^{-1} \cdot h^{-1}$) and this parameter allows the comparison of strains and experimental models. Besides, specific productivity allows the determination of the cell mass required to the fermentation step [53].

Table 4 shows the main characteristics, advantages and drawbacks of the main microorganisms that have been used to produce bioethanol from lignocellulosic biomass and its derivatives [85]. There is a wide variety of microorganisms that are able to produce bioethanol but the choice must be assessed taking into account the origin and composition of the raw material, the pretreatment and/or treatments used and the main purpose of the study [4]. *S. cerevisiae*, *S. stipitis* and *Z. mobilis* are microorganisms that ferment with a high ethanol yield associated [34, 38, 58]. However, *S. cerevisiae* and *Z. mobilis* are not capable to naturally ferment pentose sugars and *S. stipitis* has low tolerance to chemical inhibitors [58]. Currently, *S. cerevisiae* and *S. stipitis* are the microorganisms more studied and considered among those with high potential to commercial scale-up [1]. However research with thermophiles has been increasing, genetic and metabolic engineering are being widely developed, so those organisms may play an important role in the future.

Table 4 – Main characteristics, advantages and drawbacks of the most used microorganisms for bioethanol production from lignocellulosics. Adapted from *Limayem et al.* (2012) [4, 53, 85].

Microrganism	Characteristics	Advantages	Drawbacks
Saccharomyces cerevisiae	Facultative anaerobic yeast	- Naturally adapted to ethanol fermentation; - High ethanol yield; - High tolerance to ethanol and inhibitors; - Potential to commercial scale-up; - Amenability to genetic modifications.	- Not able to ferment xylose and arabinose sugars; - Not able to survive high temperature of enzyme hydrolysis.
Scheffersomyces stipitis	Facultative anaerobic yeast	- Best performance on xylose fermentation; - High ethanol yield; - Potential to commercial scale-up; - Able to ferment most of cellulosic-material sugars including glucose, galactose and cellobiose.	- Intolerant to ethanol concentrations above 40 g/L; - Does not ferment xylose at low pH; - Sensitive to chemical inhibitors; - Requires micro-aerophilic conditions to reach peak performance; - Re-assimilates formed ethanol.
Candida shehatae	Micro-aerophilic yeast	- Ferment xylose.	- Low ethanol tolerance and ethanol yield; - Require micro-aerophilic conditions; - Does not ferment xylose at low pH.
Pachysolen tannophilus	Aerobic fungus	- Ferment xylose.	- Low ethanol yield; - Require micro-aerophilic conditions; - Does not ferment xylose at low pH.
Kluveromyces marxianus	Termophilic yeast	- Able to grow at temperatures above 52ºC; - Reduces cooling cost and contaminations; - Ferments a broad spectrum of sugars; - Amenability to genetic modifications.	- High [sugars] negatively affect the ethanol yield; - Low ethanol tolerance; - Inefficient xylose fermentation, leading mainly to the production of xylitol.
Zymomonas mobilis	Ethanologenic Gram-negative bacteria	- Ethanol yield surpasses *S. cerevisiae* (97% of the theoretical); - Very high ethanol tolerance and productivity; - Potential to commercial scale-up; - Amenability to genetic modification; - Does not require additional oxygen.	- Not able to ferment xylose sugars; - Low tolerance to inhibitors; - Neutral pH range.

Microrganism	Characteristics	Advantages	Drawbacks
Escherichia coli	Mesophilic Gram-negative bacteria	- Ability to ferment both pentose and hexose sugars; - Amenability to genetic modifications.	- Repression catabolism interfere with co-fermentation; - Limited ethanol tolerance; - Restricted pH and temperature growth range; - Organic acids production; - Low tolerance to inhibitors and ethanol.
Klebsiella oxytoca	Mesophilic Gram-negative bacteria	- Ability to ferment both pentose and hexose sugars; - Naturally grows in paper and pulp streams; - Amenability to genetic modifications; - Potential to commercial scale-up.	- Low ethanol tolerance.
Thermoanaerobacterium saccharolyticum ***Thermoanaerobacter ethanolicus*** ***Clostridium thermocellum***	Extreme anaerobic termophilic bacteria	- Resistance to temperatures of 70ºC; - Ability to ferment a broad spectrum of sugars; - Continuous ethanol removal; - Amenability to genetic modification.	- Low ethanol tolerance.

Since there is no organisms satisfying all the criteria, the objective worldwide is to discover, develop or artificially create a microorganism which satisfies the requirements stated by *Balat et al.* (2011) [86]:

- Bioethanol yield: ≥ 90% theoretical;
- Bioethanol tolerance: ≥ 40 g ethanol . L^{-1};
- Bioethanol productivity: ≥ 1 g ethanol . L^{-1} . h^{-1});
- Robust grower and simple growth requirements: inexpensive medium formulation;
- Ability to grow in undiluted hydrolysate: resistance to inhibitors;
- Culture growth conditions retard contaminants: acidic pH or higher temperatures.

▪ 1.4.1.2. Bioethanol from SSLs

The investigation of new technologies and optimization of lignocellulosic by-products bioprocessing, such as SSLs, in order to produce second-generation bioethanol and other value-added compounds is one of the main concerns in energy and science field.

SSLs are daily produced in large amounts and have a high concentration of monomeric and oligomeric sugars, resulting from wood chips hydrolysis during pulping process. These factors make them potential substrates for bioprocessing and production of second-generation bioethanol [23, 33, 87]. The reduction of bioethanol production costs depends, essentially, on the purchase price of feedstock and the cost of feedstock processing. Since SSLs are by-products with low cost associated, they are good candidates for low-price fuel, large-scale production and environmental benefits [1].

Bioethanol production from SSLs has been tested in small scale by several research institutions and in some cases relatively high ethanol yields were obtained (≥ 0.30 g ethanol . g substrate^{-1}), constituting good perspectives for increased scale experiments [32, 88]. Second-generation bioethanol is not produced in a large-scale and at a competitive level yet, due to the high cost of processing with the currently available technologies [89]. The main economic challenges are the initial investment, the high amount of feedstock and the production costs [30].

1.5. Microorganisms

• 1.5.1. *Scheffersomyces stipitis*

Scheffersomyces stipitis (formerly *Pichia stipitis*) is a haploid, homothallic or heterothalic, *hemiascomycetous* and *Pseudohyphae* yeast [90-93]. Cell division is done by multilateral budding and true hyphae do not develop [93]. The genus *Scheffersomyces* contains the majority of the yeasts that are able to metabolize D-xylose, and consequently, that are of interest to biotechnology [93]. *S.*

stipitis forms yeast-like buds during exponential growth, hat-shaped spores and pseudomycelia under carbon-limited conditions in a continuous reactor. It is able to metabolize most of the sugars found in wood (glucose, mannose, galactose and cellobiose along with mannan and xylan oligomers) and to transform low-molecular weight lignin moieties [27, 32, 87, 94, 95]. This is probably due to the fact that this microorganism is closely related to yeast endosymbionts that inhabit and degrade white-rotted hardwood [96].

S. stipitis is able to metabolize several sugars, polyols, and organic acids but nitrate is not utilized [93]. *S. stipitis* is capable of efficiently use pentoses, having the highest native capacity for D-xylose fermentation of any known microbe, which is essential to reach high productivity in bioethanol production [3, 33, 93, 97-99].

S. stipitis induces fermentative activity in response to oxygen limitation, the optimal activity being reached with microaerophilic conditions (1-15% O_2) [100, 101].

Jeffries et al. (2007) sequenced and assembled the complete genome of *S. stipitis* and the sequence data revealed unusual aspects of genome organization: numerous genes for lignocellulose bioconversion, a preliminary insight into regulation of central metabolic pathways and several examples of co-localized genes with related functions [87]. This study found some aspects that might be involved in the regulation of redox balance while very efficiently fermenting xylose under microaerophilic conditions [87].

All the particular characteristics and unique physiological traits, together with the facts that *S. stipitis* has the ability to convert xylose almost exclusively into ethanol with no other significant by-products attaining one of the highest ethanol conversion rates, makes this yeast a potent and very useful microorganism for bioprocessing SSLs and other lignocellulosic residues [32, 102-104]. However, *S. stipitis* produces ethanol only if the oxygen supply is limited and it has low tolerance to ethanol [105]. Besides, pentose-fermenting yeasts generally suffer from hexoses repression, i.e. pentoses consumption rate is negatively affected by the presence of hexoses, mainly glucose and mannose. In addition, it is necessary to improve the fermentative performance in the presence of inhibitors formed during wood cooking and/or hydrolysis processes [106-109]. These drawbacks can be overcome by improving strains through genetic means or adaptation strategies [105, 110].

- **1.5.2. *Saccharomyces cerevisiae***

One of the most effective, widely studied and well-known ethanol producing microorganisms from hexose sugars is the yeast *S. cerevisiae*. It is also known as *Baker's yeas*t because of the common use on bakery industry and it is closely related with the brewing industry, having a wide public acceptance and a huge importance in several daily products [31].

Fermentative processes by *S. cerevisiae* are regulated by sensing the presence of glucose [87]. Cellular transport of glucose can be made by different transporters, with higher or lower glucose affinity, and its expression depends on glucose concentration on the media [111]. The robust wild-type (*wt*) *S. cerevisiae* is unable to efficiently utilize xylose as a sole carbon source and cannot ferment xylose into ethanol, although it can metabolize its isomer xylulose (xylose isomer). Despite the existence of glucose transporters that can transport xylose and all the subsequent enzymes needed for a full xylose metabolic pathway, *S. cerevisiae wt* expression level is very low and production rates of ethanol from xylose are significantly lower than the verified for glucose as substrate [53, 58, 59, 112].

The ethanologenic yeast *S. cerevisiae* displays high inhibitor tolerance and ethanol productivity on SSL and has been suggested as a suitable biocatalyst for SSL fermentation [113].

Several metabolic engineering and genetic modification strategies have been applied in order to enhance the efficiency of xylose fermentation to ethanol [53, 58, 59, 114]. The main objective is to create an adapted or engineered *S. cerevisiae* strain that may be able to convert glucose and xylose (main sugars in lignocellulosic by-products) with the same efficiency that hexoses are naturally metabolized [53, 59]. This improvement would lead to significant changes in bioprocesses, probably turning them economically sustainable [88].

The extensive and detailed scientific knowledge is one of the most advantages of working with *S. cerevisiae*. In fact, the possibilities of improve and increase the fermentative activity of this yeast are very wide and researchers are trying to implement the use of S. *cerevisiae* in large-scale bioprocesses. *S. cerevisiae* is considered the most promising yeast species in this scientific and industrial field [30, 39].

- **1.5.3. *S. stipitis* Vs *S. cerevisiae***

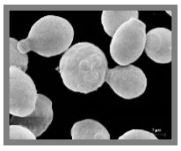
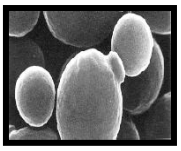
Table 5 summarizes and compares the main characteristics of *S. stipitis* and *S. cerevisiae*.

The sizes of both species are variable in a small range and *S. cerevisiae* can be up to two times bigger than *S. stipitis* [87]. This fact may be important in co-culture studies because it is possible to differentiate both species through observation on optical microscope, which is a simple and, in this case, trustable technique to use.

Hexoses fermentation efficiency is high in both yeasts but conversion rates and yields are generally higher for *S. cerevisiae* [1, 88]. However, only *S. stipitis* is able to significantly metabolize pentoses, because the genes responsible for xylose and arabinose conversion are expressed only in residual levels in *S. cerevisiae*. In *S. stipitis*, the metabolism of xylose starts with NAD(P)H-dependent xylose reductase (XR), which converts it into xylitol, and NAD-dependent xylitol dehydrogenase (XDH), that converts this alcohol into xylulose. Xylulokinase uses ATP to

form D-xylulose-5-P and the metabolic process takes place through pentose-phosphate pathway (PPP) [30, 115]. In bacteria, xylose conversion may also occur by xylose isomerase (XI) activity [53, 59].

Table 5 – Comparative analysis of the main characteristics of *S. stipitis* and *S. cerevisiae*.

	 ***S. stipitis***	 ***S. cerevisiae***
Size [87]	3-5 µm	5-10 µm
Hexoses comsuption [1, 32, 53, 87]	High	Very High
Pentoses comsuption [1, 87]	High	Very Low
Aerobic fermentation conditions [87]	Microaerophilic	Aerobic/Anaerobic
Theoretical ethanol yield [2, 59, 87]	High	High
Ethanol tolerance [2, 88]	Low	High
Acid tolerance [88]	Low	Medium
Inhibitors tolerance [88]	Low	Medium
Genome size [87]	15.4 Mb	12.1 Mb
Average gene density (%) [87]	55.9	70.3

During recent years, numerous approaches have been used to modify *S. cerevisiae* for xylose, xylan or cellulose metabolism, including genetic engineering strategies with genes from *S. stipitis* (XR and XDH) or XI related genes [53, 59, 104]. However, these transformations are only partly successful. According to *Jeffries et al.* (2009), genetically improved *S. cerevisiae* lacks too many levels of the assimilatory genes, sugar transporters and mechanisms for balancing cofactor levels when are tested under oxygen-limiting conditions [104]. More detailed information about genomics, proteomics and metabolism regulation of *S. stipitis* certainly will allow improvements and achievements on ethanol productivities from by-products [104]. A further knowledge of *S. stipitis* may also allow target genetic engineering that would improve the fermentation efficiency [115].

Adaptation strategies have also been used in order to improve *S. cerevisiae* fermentative performance. SSL-adapted pentose-fermenting yeasts generally give lower ethanol yields from SSL compared to SSL-adapted *S. cerevisiae*, even though wild-type *S. cerevisia*e cannot utilize pentoses [33, 116].

Theoretical ethanol yields vary substantially with fermentation conditions and used yeast strain, even if they are from the same species. Several studies found that *S. cerevisiae* and *S. stipitis* maximum ethanol yields, in complex media as lignocellulosic residues, are close to the theoretical maximum (0.51 g ethanol . g sugars^{-1}) [1, 88]. However, conversion rates are generally higher for *S. cerevisiae*, which leads to higher specific productivities and, consequently, to a more sustainable and economically rentable industrial bioprocesses [87].

Fermentative metabolism in *S. stipitis* needs very small amounts of oxygen for efficient xylose utilization. Although aeration favors respiration process, ethanol oxidation and re-assimilation occurs even in the presence of appreciable xylose concentrations, which may result in lower ethanol yields [59].

One of the major drawbacks of *S. stipitis* is the low tolerance to ethanol and inhibitors, that can drastically affect its performance [102]. *Skook et al.* (1992), stated that *S. stipitis* fermentation is completely inhibited in the presence of 42-45 g ethanol . L^{-1}, whereas in same conditions *S. cerevisiae* was not inhibited until 300 g ethanol. L^{-1} [117]. In microaerophilic conditions, *S. stipitis* metabolism is not affected with ethanol concentrations below 20-28 g . L^{-1} [118, 119]. Despite sensitivity to most of referred inhibitors in SSLs, when appropriate substrate purification or detoxification are previously done, *S. stipitis* showed promising results on ethanol production [33, 99, 120]. Another approach is to adapt *S. stipitis* to inhibitors and increase its tolerance to acetate, since it may cause total growth inhibition when present in higher amounts than 3 g . L^{-1} [33, 39, 42].

- 1.5.3.1. Bioethanol production from SSLs

Table 6 shows fermentation results for *S. stipitis* and *S. cerevisiae*, the microorganisms that have been the most studied to bioethanol production from SSLs. The performance of the microorganism is a function of the raw-material composition, the detoxification method, the type and geometry of the reactor, aeration, temperature and pressure. The influence of these factors is represented on Table 6, showing the effects in terms of ethanol efficiency production (yield and volumetric productivity).

The fermentation performances of *S. stipitis* and *S. cerevisiae* in untreated SSLs are strongly affected, since the ethanol yields obtained correspond only to about one 30-50% of the theoretical maximum [33, 116].

Detoxification has a positive effect on the ethanol production. *S. stipitis* and *S. cerevisiae* reach higher ethanol yields when a process is applied in order to remove some of the chemical inhibitors present in SSLs [27, 32, 33, 37].

As stated before (topic 1.3.3.1.), yeast strain adaptation is one of the used strategies to improve the alcoholic fermentation efficiency [73]. Usually, the adaptation consists in submitting the strains to similar stress conditions that are present on the raw material, in this case on the SSLs. This selection shall enhance the growth of a more resistant population. An efficient adaptation and selection can improve by up 2-fold the maximum ethanol yield [121]. *S. stipitis* is a good example where detoxification is a successful approach, since it led to an increase on the ethanol yield from 0.16 to 0.28 in untreated HSSL and from 0.30 to 0.41 in HSSL pretreated by overliming, respectively [33]. Besides, the effect on volumetric productivity is even stronger, leading to an increase of 7-fold and 4-fold on the untreated and pretreated HSSL, respectively [33].

Metabolic and genetic engineering have been mostly applied to *S. cerevisiae* in order to enable it to ferment D-xylose and L-arabinose to ethanol. However, *S. stipitis* has also been target of this engineering approach to improve its native capacity of fermenting lignocellulosic feedstock to ethanol [121]. *S. cerevisiae 259ST* is a GMO used for bioethanol production, whose genetic engineering resulted in an improvement of ethanol yield (2,5-fold) and productivity (3-fold), comparing to the original strain.

Cell immobilization has been presented as an advantageous approach to ferment lignocellulosic wastes with inhibitors, improving cell stability, cell activity and cell stress tolerance [122]. The immobilization techniques for yeasts can be classified in four categories: attachment or adsorption to solid surfaces (wood chips, DEAE cellulose, porous glass); entrapment within a porous matrix (calcium alginate, k-carrageenan, agar, chitosan, etc); mechanical retention behind a barrier (microporous membrane filters and microcapsules); and self-aggregation of the cells by flocculation [123, 124]. These different immobilization methodologies have been largely discussed in order to understand and evaluate the impact in the microbial growth and physiology, internal and external mass transfer limitations, bioreactor design, bioprocess engineering and economics [123, 125].

Table 6 – Fermentation parameters of SSLs using different microorganisms.

Microorganism	SSL	Detoxification method	$Y_{P/S}$ (g .g $^{-1}$)	P_E (g. L^{-1}.h^{-1})	Ref.
S. stipitis NRRLY-7124	HSSL	Untreated	0.16	0.01	[33]
		Biological treatment w/ *P. variotii*	0.24	0.09	[27]
		Overliming	0.30	0.11	[33]
		Ion exchange resins	0.49	1.22	[32]
S. stipitis [a]	HSSL	Untreated	0.28	0.07	[33]
		Overliming	0.41	0.44	[33]
S. stipitis R [b]	SSSL	Steam-stripping	0.42	1.30	[126]
S. cerevisiae 259 A	Mixture	Untreated	0.15 - 0.32	0.03	[116]
S. cerevisiae R57 [a]	HSSL	Untreated	0.15 - 0.32	0.03	[116]
S. cerevisiae T2 [c]	Mixture	Untreated	0.41	*	[127]
S. cerevisiae RLJY - 019 [a]	HSSL	Overliming	0.47	0.45	[33]
S. cerevisiae 259ST [d]	Mixture	Untreated	0.32 - 0.42	0.10	[116]

[a] - HSSL adapted strain
[b] - Immobilized yeast
[c] - SSL adapted strain
[d] - GMO (Genetically Modified Organism)
* - Data not available

S. stipitis R (Table 6) was immobilized and tested for SSSL fermentation and it led to an ethanol yield above 90% of the theoretical maximum. However, the most significant effect was on the volumetric productivity, the highest in Table 6, which means that this specific strain and strategy were successful [126]. Several theories have been proposed to explain this kind of improvement on the fermentation performance when immobilization is applied [123]. In the case of entrapment method, it was suggested that the layer creates a microenvironment that acts as a protection for the chemical inhibitors. Besides, if the microorganism needs oxygen limited conditions to achieve the optimal ethanol yield, as *S. stipitis*, the layer might act as a natural oxygen controller since it hinders the transfers between external and internal parts. When a physical support is used for immobilization the adsorption of ethanol and inhibitors by the support may also act to minimize the inhibition [123, 128]. The product recovery, the possibility to reuse the biomass, the increase of substrate uptake, the improvement of ethanol yield and the lower risk of microbial contamination are other advantages of immobilization that compensates the additional investment that this process involves [125].

The detoxification methods, the adaptation of strains to stress conditions present on SSLs, the metabolic and genetic engineering and immobilization techniques are different strategies that have been widely studied over the last decades. Each one ends in a different effect on fermentation process and/or in the end-product but all of them lead to improvements on the ethanol yield and volumetric productivity. The simultaneous utilization of more than one of these techniques/strategies is the next step to improve the process and its efficiency, in order to reach economic and environmental sustainability and stability.

2. Methods and Experiments

2.1. Microorganisms and Maintenance

Scheffersomyces (Pichia) stipitis NRRL-7124 was gently supplied by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA.

Saccharomyces cerevisiae TMB 3720 is an industrial strain isolated from SSL plant (*Sanchez Nogue*, *personal communication*). A second industrial *Saccharomyces cerevisiae* strain, TMB 3500 was also used in this study [129].

The stock cultures of the strains were stored in 30% glycerol at -80^{o}C. *S.stipitis* culture was grown at 30^{o}C and maintained in YM plates at 4^{o}C. *S. cerevisiae* strains were cultivated in YPD plates at 30^{o}C and maintained at 4 ^{o}C.

2.2. Media and Stock Solutions

- **2.2.1. Culture Media**
  - 2.2.1.1. YPD (Yeast Extract Peptone Dextrose - Liquid and Plates)

	YPD Liquid	***YPD Plates***
Compound	**(g . L^{-1})**	**(g . L^{-1})**
Peptone From Casein	20.0	20.0
Glucose	20.0	20.0
Yeast Extract	10.0	10.0
Agar-agar	-	15.0

  - 2.2.1.2. YM (Yeast Medium - Liquid and Plates)

	YM Liquid	***YM Plates***
Compound	**(g . L^{-1})**	**(g . L^{-1})**
Glucose	10.0	10.0
Peptone from Casein	5.0	5.0
Malt Extract	3.0	3.0
Yeast Extract	3.0	3.0
Agar-agar	-	20.0

- 2.2.1.3. Supplemented YNB

	YNBglc Liquid	*YNBglc Plates*	*YNBxyl Plates*
Compound	**(g . L^{-1})**	**(g . L^{-1})**	**(g . L^{-1})**
Glucose	20.0	20.0	-
Xylose	-	-	20.0
Yeast Nitrogen Base w/o Aminoacids	6.70	6.70	6.70
Potassium hydrogen phthalate	10.2	-	-
KOH	2.2	-	-
Agar-agar	-	12.0	12.0

- **2.2.2. SSSL/HSSL Sugars Solutions**

	5x Domsjö Hyd. Sugars	*5x HSSL Sugars*
Compound	**(g . L^{-1})**	**(g . L^{-1})**
Mannose	*	42.5
Xylose	*	123
Glucose	*	11.5
Galactose	*	22.5
Arabinose	*	39.0

* SSSL detailed composition cannot be disclosed for confidential results. Therefore, the composition of the SSSL sugar stock solution cannot be presented.

- **2.2.3. Buffers**

	YNB Buffer 5x	*YNB Buffer 10x*	*10x YNB*
Compound	**(g . L^{-1})**	**(g . L^{-1})**	**(g . L^{-1})**
Potassium Hydrogen Phtalate	51.0	102	-
KOH	11.0	22.0	-
Yeast Nitrogen Base w/o Aminoacids	-	-	67.0

- **2.2.4. Salts Solutions**

	0.9 % NaCl	*EDTA 0.5 M (pH 8)**
Compound	**(g . L^{-1})**	**(g . L^{-1})**
NaCl	9.0	-
$C_{10}H_{14}N_2Na_2O_8$. 2 H_2O	-	186.1

*The pH was adjusted with NaOH 5M

- **2.2.5. Stock Solutions**
  - 2.2.5.1. Glycerol 30% (v/v)

	30 % Glycerol
Compound	**V (mL/100mL solution)**
Glycerol	30.0
mQ H_2O	70.0

2.3. SSLs

- **2.3.1. Bio-detoxified HSSL**

Caima undetoxified HSSL (Table 2) results from the magnesium-based *Eucalyptus globulus* wood cooking process, and was gently supplied by CAIMA, S. A. (Portugal). HSSL was collected from the evaporator 7, after few evaporation (Figure 2).

HSSL bio-detoxification process with the fungus *Paecilomyces variotii* was carried out in a SBR (Sequential Batch Reactor), as previously reported by *Pereira et al.* (2011) [27].

- **2.3.2. Domsjö Hydrolysate**

Domsjö hydrolysate was gently provided by Domsjö Fabriker AB (Sweden). It is produced from disodium-based wood cooking of a controlled mixture of Spruce (*Picea abis*) and Pine (*Pinus sylvestris*). The pH of Domsjö Hydrolysate was adjusted to 5.5 with KOH (powder and highly concentrated solutions).

2.4. Yeast Cultures

- **2.4.1. Inoculum**

The inocula of *S. stipitis* were prepared taking a single colony from the YM plate stored at -4^{o}C to YNBglc. Depending on the further experiments, either 5 mL of supplemented YNB in a 50

mL conical tube or 25 mL of media in a 250 mL baffled flask were used. A similar procedure was followed for *S. cerevisiae* strains and single colonies were taken from YPD plates. The incubation processes were performed at 30°C and 180 RPM during different periods for each strain in order to reach the beginning of the stationary phase.

2.5. Screening Assays

The pre-cultures were grown in 50 mL Falcon tubes as mentioned in section 2.4.1.. After pre-cultures, each strain was inoculated into six different percentages of HSSL and SSSL at an initial OD_{620nm} of 0.5. The different percentages were prepared as showed in Table 7 for each liter of SSSL or HSSL. Growth curves were performed in 5 mL of media in 50 mL conical tubes, which were incubated at 30°C and 180 rpm. The cell density was measured over time at 620nm. pH was measured at the end of the experiments. Each condition was performed in duplicate.

Table 7 - Composition of the different used percentages of SSSL and HSSL in the screening assays.

Gradient (%)	SSSL/HSSL (mL)	YNB Buffer 10x (mL)	10x YNB	SSSL/HSSL 5x Sugars Solution (mL)	mQ H_2O (mL)
0	-	100	100 mL	200	600
20	200	100	100 mL	160	440
40	400	100	100 mL	120	280
60	600	100	100 mL	80	120
80	800	100	0.67 g	40	80
100	1000	100	-	-	-

The μ_{max} values were determined considering the slope of the exponential phase of the yeast growth curves (ln OD_{620nm} Vs Fermentation time). The final values resulted from the calculation of the average and standard deviation among the duplicates performed for each condition.

From the results obtained in the screening assays, 40% of Domsjö hydrolysate and 60% of bio-detoxified HSSL were the percentages of each SSL selected to perform the wet assays (section 2.6) and to use as a pre-adaptation step in the fermentation processes.

2.6. Wet Assays

Pre-cultures of yeast strains grown in YNBglc media were used to inoculate 25 mL of 40% Domsjö hydrolysate or 60% of bio-detoxified HSSL with an initial OD_{620nm} of 0.5. Cells were

incubated at 30°C until beginning of the stationary phases was reached and the following determinations were performed:

- **2.6.1. CFUs/mL**
  - **Evaluation**

- Take an aliquot of 2.0 mL of the final screening assay tube to a sterilized *eppendorf*;
- Make the serial dilution with the autoclaved glass tubes;
  - Homogenize the content of the *eppendorfs* with a vortex;
  - Take 0.5 mL of the aliquot into the first glass tube with 4.5 mL of sterile 0.9% NaCl solution;
  - Homogenize the content of the first glass tube and take 0.5 mL into the second glass tube;
  - Repeat the process until the pretended dilutions;
- Plate 100 µL of the dilution of interest in fresh rich media plates (YM for *S. stipitis* and YPD for *S. cerevisiae* strains);
- Repeat the process two times, using a total volume of 1.5 mL and having 3 replicas for each dilution;
- Incubate the plates for two days at 30°C;
- Count the colonies in each plate.

The experimental protocol mentioned above and presented in Figure 6 was made at least in triplicate for each strain in the two different media (40% SSSL or 60% HSSL).

- **2.6.2. Wet Weight/mL**
  - **Evaluation**

- Place the remaining final fermentation media (≈23 mL) into a previously tared (P_0) and sterilized 50 mL conical tube;
- Centrifuge 5 min, 4000 RPM;
- Remove the supernatant and wash with 50 mL of 0.9% NaCl. Repeat the centrifugation step;
- Remove all the supernatant very carefully;
- Measure the weight – P_f (g);
- Determine [P] (g Wet Weight . mL^{-1}) = $P_f - P_0$.

The experimental protocol mentioned above was made at least in triplicate for each strain in the two different media (40% SSL or 60% HSSL).

With the values of the CFUs . mL^{-1} and Wet Weight . mL^{-1} it was possible to determine the ratios CFUs . $(Wet\ Weight)^{-1}$ for all the tested conditions. The ratios were needed to start the fermentations with the required number of CFUs per mL. The transfer of the cells by the OD_{620nm} method was not possible when SSLs were used, since the dark color of both SSLs interfered in the cell density measurements. The wet assays were not based in the media color, allowing the correct measurement of the cells to start the fermentations.

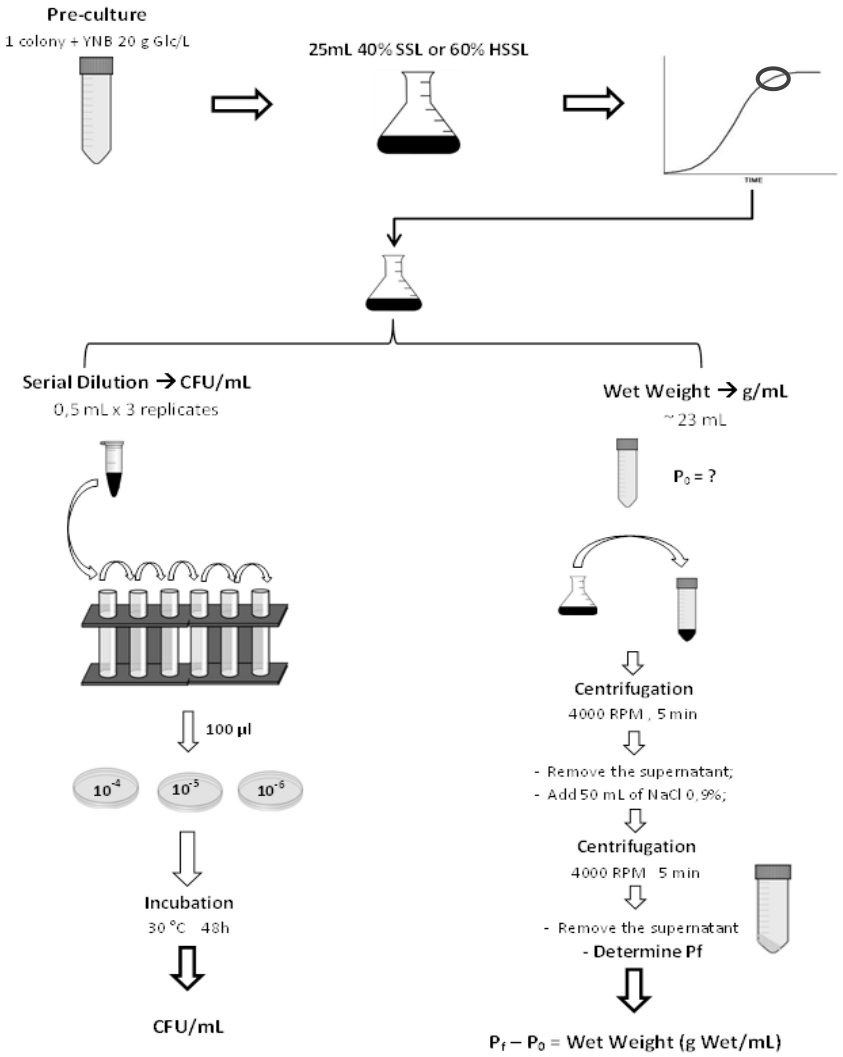


Figure 6 – Schematic view of the experimental protocol used for the Wet Assays.

2.7. Fermentations with free-cell cultures

Pre-cultures of *S. stipitis* and *S. cerevisiae* TMB 3500 were grown in YNBglc. Depending on the amount of cells needed for further steps, the pre-cultures were performed in 50 mL sterilized

conical tubes or 250 mL sterilized baffled flask. Pre-cultures were used to inoculate 100 mL of 40% Domsjö hydrolysate or 60% of bio-detoxified HSSL at an initial OD_{620nm} of 0.5. At the beginning of the stationary phase cell pellet was obtained to inoculate undiluted hydrolysate at initial cell density of 1.08×10^8 cells . mL^{-1} - level that is usually applied in bioprocessing industries.

- **Experimental protocol**
  - Pour the content of the 1000 mL flasks into a 500 mL centrifuge bottle previously tared - P_0 (g);
  - Centrifuge 5 min, 4000 RPM;
  - Remove the supernatant and wash with 0.9% NaCl. Repeat the centrifugation step;
  - Remove all the supernatant very carefully;
  - Measure the final weight – P_f (g);
  - Determine [P] (g Wet Weight . mL^{-1}) = $P_f - P_0$;
  - Using the ratio determined in the Wet Assays, calculate the wet weight that is needed to start the fermentation;
  - Resuspend the pellet with 0.9% NaCl and remove the amount media that exceed the required number of cells;
  - Centrifuge 5 min, 4000 RPM. Remove all the supernatant very carefully;
  - Resuspend the pellet with 400 mL of 100% Domsjö Hydrolysate or 100% bio-detoxified HSSL.
  - Transfer to the homemade fermentors, add 20 µL of anti-foam and start the fermentation.

Fermentations were carried out at 30^oC, initial pH of 5.5, airflow of 0.4 L air . min^{-1} through a tube with a spreader in the end, and controlled and constant agitation with a sterilized magnetic stirrer. OD_{620nm} and pH samples were taken over time. Aliquots were filtered with disposable cellulose acetate filters (0.20 µm) and supernatant were frozen at -20^oC to be further analyzed. First and last samples of each fermentation were also used to determine the initial and final CFUs. Replica plating technique was used at the end of the fermentations to verify the presence of possible contaminant microorganisms.

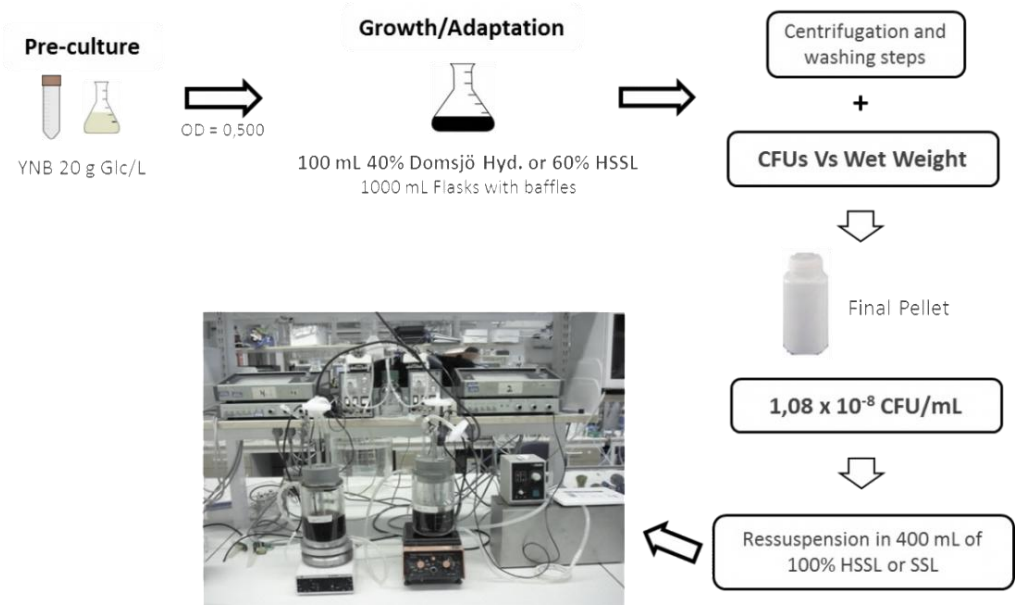


Figure 7– Scheme of the experimental protocol used for the Fermentations.

2.8. Fermentations with immobilized cultures

S. stipitis cells were immobilized by entrapment in calcium alginate gel beads. To obtain the correct amount of cells to start the HSSL fermentation, approximately 1.08×10^8 cells . mL^{-1}, the protocol was the same that the one used for the fermentations with suspended cultures until the resuspension with a SSL. The cell wet pellet was gently mixed with 45 mL of 2.8% sodium alginate. When the mixture was completely homogenized, it was added to 2.0% calcium chloride ($CaCl_2$) with sterile needles and a distance needle-solution of 4-7 cm. The beads were maintained in this solution for 20 minutes with gently agitation. Then, the gel beads were transferred into 0.5% $CaCl_2$ and maintained for 20 minutes with high agitation. All the solutions and material were previously sterilized. After the immobilization process, the *S. stipitis* beads were placed into the homemade fermentors to initiate the fermentation. When immobilization was tested, the bio-detoxified HSSL was previously supplemented and mixed with $CaSO_4$ (2 g . L^{-1}) in order to avoid the disruption of the calcium-alginate matrix by the compounds present in the liquor.

The conditions and analyzed parameters were the same that for the fermentations of suspended cultures. The analysis of the CFUs in the immobilization test included an extra step since the remaining cell beads needed to be disrupted to proceed to the plating step. In the end of the fermentation, the beads were separated from the bio-detoxified HSSL with a filter and then placed into a volume of 2% (m/v) sodium citrate solution that is determined by the formula:

$$Volume_{Sodium\ citrate} = 4.5 \times Volume_{beads\ produced\ at\ T0}$$

After beads disruption, the plating and further steps processes took place as mentioned before.

2.9. Fermentations with pH control at 5.5

Fermentations with *S. stipitis* in free-culture and immobilized in a calcium-alginate matrix were performed in bio-detoxified HSSL, maintaining the pH at 5.5. KOH 3M or H_2SO_4 3M were pumped by an automatic control system to fermentative processes in order to maintain the pH between 5.2 and 5.8 (optimal at 5.5).

Apart pH control, the steps to initiate the fermentation with free-culture and immobilized culture were those stated in topics 2.7 and 2.8, respectively.

2.10. Replica Plating

The last sample of each fermentation was plated into a YNBglc plate (primary plate) with the dilution factor to obtain single colonies. After 48 hours of incubation at 30^{o}C, cells were replica-plated into a YNBxyl plate (secondary plate). The primary plates are pressed onto a cylindrical structure covered with sterile velvet and when lifted off the cloth contains an imprint from each colony. Then, the imprint is stamped on the secondary plate and incubated for 48 hours at 30^{o}C. After this period it is possible to compare the growth of the colonies in both plate and verify which ones are able to grow on xylose.

2.11. Substrates and by-products analysis by HPLC

Two HPLC systems (Lund and Aveiro) were used in order to determine the concentration of the different compounds present during fermentations. An absolute calibration was applied for all analyzed compounds in both systems.

In Lund, concentrations of glucose, xylose and xylitol were analyzed by HPLC, using a RPM-Monosaccaride ion-exchange column (Phenomenex, CA, USA) with a mobile phase of mQ H_2O at a flux of 0.5 mL . min^{-1} and 45^{o}C. Ethanol, glycerol, acetate, HMF and furfural concentrations were determined also by HPLC, but using an Aminex HPX-87P column (Bio-Rad, USA) with H_2SO_4 5 mM at 0.6 mL . min^{-1} as mobile phase and 85^{o}C. The injected volume was 20 µL. Both HPLC systems were equipped with Waters HPLC pump, autosampler (Waters 717 plus),

CH-30 Column Heater (FIAtron™ Systems Inc.), Shimadzu Refractive Index Detector RID-6A and absorbance detector (Waters 2487 Dual λ).

In Aveiro, concentrations of glucose, xylose, ethanol, acetate, xylitol and glycerol were determined by using a Eurokat H 10 µm 300x8 column (Knauer) with H_2SO_4 0.01N at 0.4 mL . min^{-1} as mobile phase and 40ºC. The injected volume was 20 µL. HPLC system was equipped with a Merck Hitachi Pump L-2130, autosampler L-2200 (Merck Hitachi), oven Gecko 2000 set at 40 ºC and refraction index (RI) detector L-2490 (Merck Hitachi).

2.12. *S. stipitis* beads analysis

- **2.12.1. Fixation, Dehydration and Embedding**

In order to determine the distribution of cells inside the calcium alginate beads, thin transverse sections were required for direct microscopic observation. These thin sections were obtained by a sequential process that included fixation, dehydration and embedding steps.

The fixation started by placing the cell beads on tubes with 2.5% glutaraldehyde in cacodylate buffer 0.1M and protected from light during 2 hours. Then rinsing with cacodylate buffer 0.1M was made. After 20 minutes, the dehydration started using sequential and increasing ethanol solutions and different periods: 30%, 50% 70 for 20 minutes, 96% for 10 minutes, 100% and 100% for 20 and 30 min, respectively. After removing the last ethanol solution from the tubes, propylene oxide (P.O.) was added for 10 minutes, twice. All these steps were done at 4ºC.

The embedding process started with a mixture P.O. + Spurr (1:1) for 14-17 hours. Then, beads were transferred twice into fresh Spurr for 4-5 hours and 20-22 hours. This process was done in a rotatory shaker at 22-23ºC and 100 RPM.

Finally, the dehydrated beads were placed in inclusion molds of silicone with 100% Spurr in order to obtain the blocks to do the microtome cut of the beads. The Spurr (Fluka) polymerization took place during 12-15 hours at 80ºC.

- **2.12.2. Microtome cut**

In order to evaluate the distribution of cells inside the calcium alginate beads, thin transverse sections were required for direct microscope observation. After the polymerization, the specimen blocks were cut into sections of 1.5 µm thickness by means of a Leica EM UC6 ultramicrotome equipped with glass blades.

- **2.12.3. Microscope Observation and Analysis**

The sections obtained by the microtome cut were stained with toluidine blue in order to highlight the presence of cells. After this step, the sections were examined by phase contrast microscopy, using a Zeiss Axioskop optical microscope equipped with Zeiss Axicam HRc digital camera. The pictures were taken with 100× and 400× magnifications.

3. Results and Discussion

3.1. Screening Assays

The determination of μ_{max} values for each strain in six different percentages (0, 20, 40 60, 80 and 100%) of bio-detoxified HSSL and Domsjö hydrolysate was the starting point of this comparative study. The results showed in Figure 8 allow the direct comparison of three different variables: yeast strain, SSL type and SSL percentage. Both SSLs were compensated for sugars and salts in order to avoid nutrient limitation. Thus, possible changes on growth were caused only by the presence of inhibitory chemical compounds.

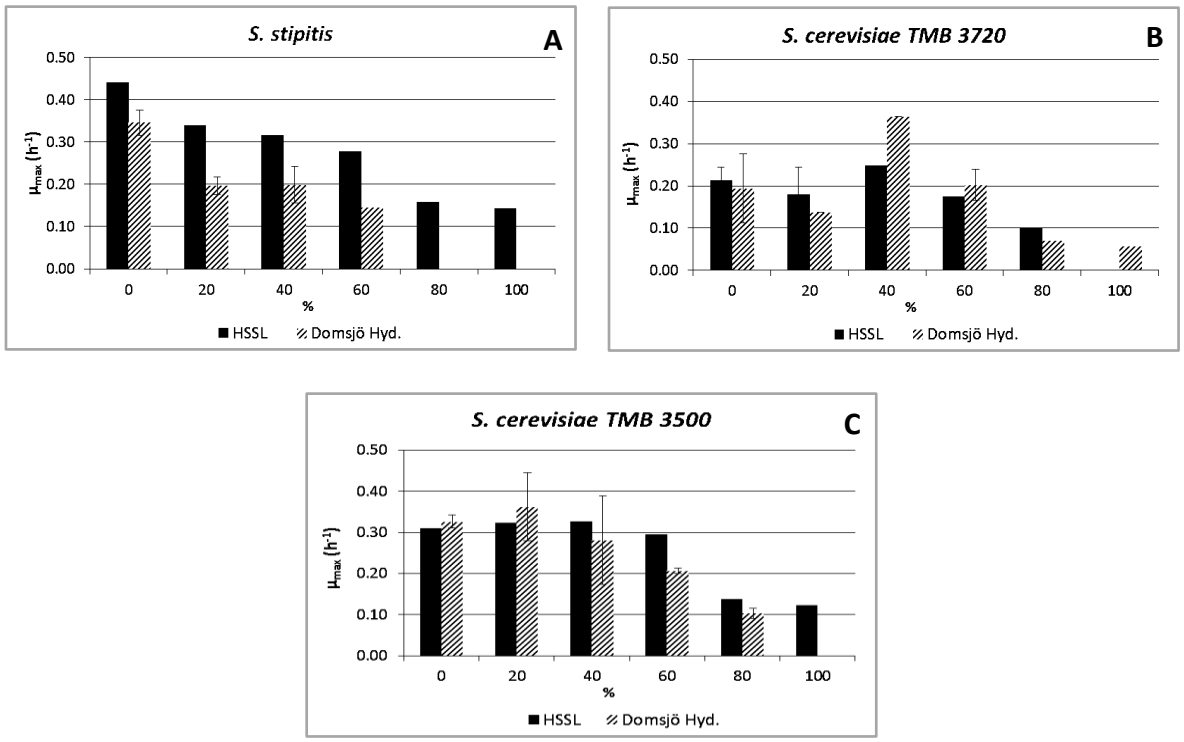


Figure 8 - Maximum specific growth rate of *S. stipitis* (A), *S. cerevisiae* TMB 3720 (B) and *S. cerevisiae* TMB 3500 (C) for different percentages of HSSL (filled rectangles) and Domsjö hydrolysate (striped rectangles).

Without SSL, the μ_{max} obtained for *S. stipitis* were very similar and consistent to the values related in literature. *Xavier et al.* (2010) found that the maximum specific growth rate of this specific yeast strain was 0.37 h^{-1}, result that is in agreement with those showed in figure 8-A [32].When 20% was tested, μ_{max} decreased to 0.34 h^{-1} in HSSL and 0.20 h^{-1} in Domsjö hydrolysate. These results were expected since inhibitors content increased and Domsjö hydrolysate is a less suitable medium than HSSL due to the lack of a previous bio-detoxification step. Maximum specific growth rates decreased very slightly from 20 to 60% in both SSLs, even with the relatively high increase of inhibitors concentration. Concerning HSSL, μ_{max} was around 0.30 h^{-1} and in

Domsjö hydrolysate was slightly lower (≈ 0.20 h^{-1}) in 20, 40 and 60%. SSLs percentages above 60% had a significant negative impact on *S. stipitis* growth. Although *S. stipitis* has demonstrated ability to grow in 80 and 100% of bio-detoxified HSSL, growth rates were very low (≈ 0.15 h^{-1}), nearly one third of μ_{max} obtained in non-inhibitory conditions. In Domsjö hydrolysate, *S. stipitis* did not show ability to grow on the two higher percentages even after 100 hours of cultivation, which suggests a complete inhibition of the yeast metabolism by the inhibitor content.

When *S. cerevisiae* TMB 3500 was tested, no significant changes on μ_{max} were detected until 60% of both SSLs (figure 8-C). With HSSL, values were very stable and close to 0.30 h^{-1}, and with Domsjö hydrolysate there was some slight variation and μ_{max} varied between 0.22 and 0.35 h^{-1}. As in the case of *S. stipitis*, growth of *S. cerevisiae* TMB 3500 was severely affected with 80 and 100% of SSLs. In 80% of SSLs, the yeast was able to grow but at much less extent than in lower percentages, since μ_{max} values were very low (≈ 0.10 h^{-1}). When 100% of bio-detoxified HSSL was used, *S. cerevisiae* TMB 3500 grew in the same order of magnitude as on 80% (≈ 0.10 h^{-1}). Hence, the biggest reduction in the maximum specific growth rate occurred between 60 and 80%. *S. cerevisiae TMB 3500* did not grow in 100% of Domsjö hydrolysate due to the presence of a high concentration of inhibitors.

These results from the screening of both SSLs suggest that *S. cerevisiae* TMB 3500 is more robust and tolerant than the other two tested strains because μ_{max} were similar in both SSLs, except in 100%. Thus, TMB 3500 strain may have response mechanisms to the presence of inhibitory compounds that have no negative effect until threshold concentrations are achieved.

Analyzing figure 8-B, it is possible to conclude that results with *S. cerevisiae* TMB 3720 were very different than for the two other yeast strains. In this case, maximum values of μ_{max} were obtained with 40% of SSL and not in the absence of inhibitors. Thus, metabolic detoxification mechanisms or some enzymes that confer resistance to inhibitory compounds might have optimal activity with specific amounts of inhibitors present in the medium. In total absence of inhibitory compounds, these pathways that are also related with growth ability might be repressed, negatively affecting the yeast metabolism. Another unexpected result was the ability of *S. cerevisiae* TMB 3720 to grow on 100% of Domsjö hydrolysate, although with a low μ_{max}, and the lack of growth in the same percentage of bio-detoxified HSSL, that contains less inhibitors. This fact reinforces the hypothesis that this microorganism might reach the optimal growth activity only when a specific concentration and type of inhibitors are present. This optimal inhibitors concentration must be below threshold level.

Screening assays allowed a comparative analysis of the three yeast strains in two different SSLs. Results showed that 60% bio-detoxified HSSL and 40% Domsjö hydrolysate were the best percentages to have a good compromise between the final obtained biomass (proportional to

OD_{620nm}), the maximum specific growth and the ability to successfully resist to the presence of yeast metabolism inhibitors. Hence, these percentages of each SSL were used in the following experiments, because they were the maximum content of both SSLs that did not significantly affected yeast growth. These SSLs percentages were used as a pre-adaptation step in order to enhance fermentation efficiencies in undiluted HSSL and SSSL [33, 130].

3.2. Wet Assays

The wet assays were performed in order to obtain a relationship between the yeasts wet weight (g wet . mL^{-1}) and the CFUs (CFUs . mL^{-1}) in 40% Domsjö hydrolysate or 60% bio-detoxified HSSL, since it was not possible to obtain the traditional calibration curve for cell concentration based on the OD_{620nm} measurements. With this ratio it would possible to reach the required CFUs to start the fermentation (1.08×10^8 CFUs . mL^{-1}) from the measure of the wet weight. The cells number of 1.08×10^8 CFUs . mL^{-1} was chosen because the bio-industries use a concentration of yeast colonies in this order of magnitude to start the bioprocess. Thus, the wet weight allowed the measure of the required CFUs and maintained the viability of the yeast cells.

Table 8 shows the obtained ratios of CFUs and wet weight for the three tested strains in the SSLs percentages chosen with the screening assays. Both SSLs contain a considerable amount of solids, which means that even in the absence of yeast there is a wet weight associated to each percentage of SSLs. Since HSSL was submitted to a physico-chemical pretreatment and a biological detoxification, it contains a lower amount of solids. Thus, a similar ratio of CFUs per grams of wet weight should correspond to a lower amount of cells in the case of Domsjö hydrolysate.

S. stipitis and *S. cerevisiae* TMB 3500 showed identical results in each SSL: 12×10^9 and 11×10^9 in bio-detoxified HSSL and 7.0×10^9 and 6.8×10^9 CFUs . (g wet weight)$^{-1}$, respectively. Besides, the values obtained in 40% Domsjö hydrolysate were about half of those obtained in 60% HSSL, for both strains. These results point that *S. stipitis* and *S. cerevisiae* TMB 3500 behaved similarly on tested conditions and determined ratios were reproducible because standard deviation were relatively low.

In opposition to the observed results of *S. stipitis* and TMB 3500, the *S. cerevisiae* TMB 3720 ratio was higher in Domsjö hydrolysate, which is also in agreement with the screening assays. Using the same fermentation time, *S. cerevisiae* TMB 3720 produced less number of cells and a higher wet weight, which leads to lower ratio values.

Table 8 - Ratios between the number of colonies (CFUs . mL^{-1}) and the wet weight (g . mL^{-1}) obtained in 40% Domsjö Hydrolysate and in 60% bio-detoxified HSSL.

	S. stipitis	***S. cerevisiae* TMB 3720**	***S. cerevisiae* TMB 3500**
40% Domsjö Hydrolysate (CFUs . (g Wet × 10^9)$^{-1}$)	7.0 ± 2	6.3 ± 2	6.8 ± 3
60% Bio-detoxified HSSL (CFUs . (g Wet × 10^9)$^{-1}$)	12.3 ± 2	1.4 ± 1	11.5 ± 2

S. cerevisiae TMB 3720 was the only tested yeast strain that flocculated. This feature should also be related with the differences observed, because it is much more difficult to manipulate yeasts with this characteristic. Wet assays experiments with this yeast were repeated at least 5 times, however standard deviations were very high and values not completely trustable, which suggests that the techniques used for this experiments should be changed and/or optimized in order to guarantee that flocculation is a factor that do not negatively affect results reproducibility.

After choosing the best percentages of HSSL and Domsjö hydrolysate that were possible to bioprocess without affecting yeast metabolism, wet assays allowed the choice of the two best strains for fermentation assays. Considering the ability to grow, the reproducibility of the methods and the values obtained, *S. stipitis* and *S. cerevisiae* TMB 3500 were chosen to perform the fermentations in undiluted HSSL and Domsjö hydrolysate.

3.3. Fermentations

Batch test fermentations were performed in 800-1000 mL homemade fermentors with a working volume of 40% of the total volume, in duplicate. Initially, four different conditions were tested: *S. stipitis* in 100% Domsjö hydrolysate, *S. cerevisiae* TMB 3500 in 100% Domsjö hydrolysate, *S. stipitis* in 100% bio-detoxified HSSL and *S. cerevisiae* TMB 3500 in 100% bio-detoxified HSSL. Then, in a second-stage of this project, the effects of cell immobilization and pH control were studied with *S. stipitis* on bio-detoxified HSSL.

• 3.3.1. Free-cell assays with 100% Domsjö hydrolysate

Figure 9 presents the time course experiment for the batch test performed with *S. cerevisiae* in Domsjö hydrolysate. This figure shows that biomass was approximately stable during fermentation while ethanol was produced. It can also be seen that xylose and acetate were not consumed and that xylitol, furfural and HMF were in residual concentrations or below the detection

level during this assay. Glucose minimum concentration and ethanol highest concentration (18.7 g . L^{-1}) were achieved after 15 hours and 19 hours of fermentation, respectively. Acetate content was stable during this period, meaning that it was not assimilated. Therefore, another hexose (probably mannose) was probably consumed and responsible for this ethanol increase. Unfortunately mannose was not quantified by HPLC. At the same moment that ethanol production started, glycerol was also produced and reached 5.5 g . L^{-1} at 19 h, level that was stable until the end of the experiment. The substrate was metabolized into a product beyond ethanol, which means that ethanol production could be more efficient if decreasing the glycerol production.

Aerobic conditions led to the consumption of ethanol after its maximum production at 19 hours, because *S. cerevisiae* TMB 3500 used it as carbon source after the depletion of hexoses. This fact reduced the efficiency of ethanol yields and productivities. However, it was unexpected that ethanol started to be consumed with the presence of glucose, since this hexose is the simplest compound that can be used as carbon source. This result suggests the existence of a mechanism that blocked the consumption of glucose. HMF and furfural were not present in sufficient amounts to strongly inhibit fermentation.

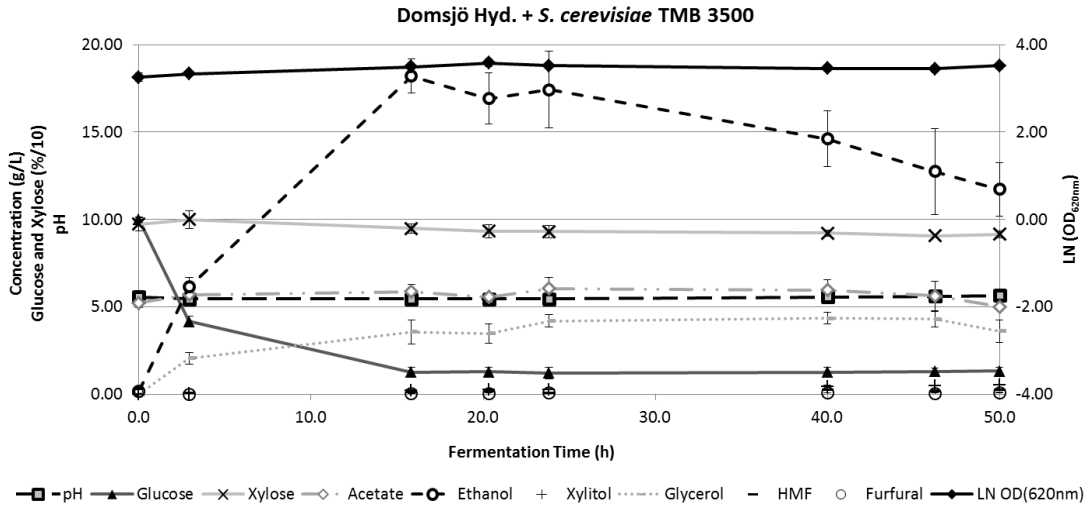


Figure 9 - Glucose, xylose, xylitol, glycerol, acetate, ethanol, HMF and furfural concentration profiles using *S. cerevisiae* TMB 3500 in 100% Domsjö Hydrolysate. The represented sugars % is a tenth of the real one, meaning that 10.00 correspond to 100%.

Unfortunately, contaminations on the batch assay performed with *S. stipitis* in 100% Domsjö hydrolysate were detected twice through observations in optical microscope. It was verified the presence of another yeast, probably *S. cerevisiae*, and replica plating confirmed the result. Thus, results for these experiments are not shown.

- **3.3.2. Free-cell assays with 100% bio-detoxified HSSL**

Figure 10 compares the concentration profiles of several compounds metabolized or produced by *S. stipitis* (Figure 10-A) and *S. cerevisiae* TMB 3500 (Figure 10-B). With both yeasts, very low levels of xylitol, HMF and furfural were observed and, in some cases, they were below the HPLC detection limit. For both species the pH remained stable during the first 25h of fermentation and then it started to slightly increase until 50h, causing a difference of 0.8 to 1.2 as compared to the initial value.

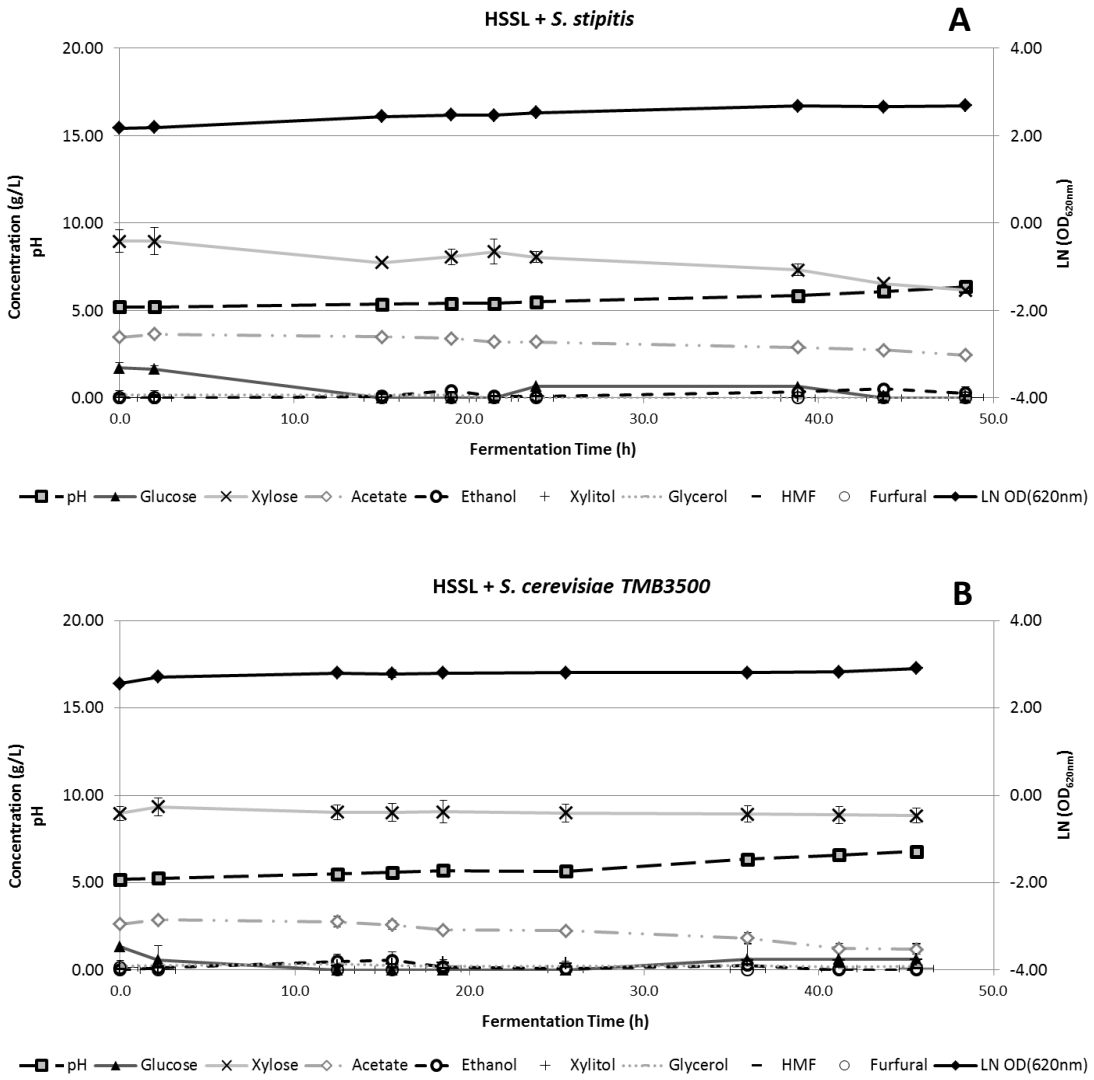


Figure 10 - Glucose, xylose, xylitol, glycerol, acetate, ethanol, HMF and furfural concentration profiles using *S. stipitis* (10-A) and *S. cerevisiae* TMB 3500 (10.B) in 100% bio-detoxified HSSL.

During *S. stipitis* fermentation, glucose and xylose were simultaneously consumed at similar rates. Glucose was depleted after 25 hours. Thereafter, acetate, that was present below inhibitory concentration (since there was no lag phase), started to slightly decrease, acting as another carbon

source. Xylose was not completely depleted and the consumption of organic acids and alcohols was unexpected, since there were available monomeric sugars. Ethanol concentration was above 0.4 g . L^{-1} at 19h and also 44h but in between decreased, probably due to the presence of oxygen in the fermentor, allowing the consumption of ethanol. After 44 h of fermentation, maximum ethanol concentration was achieved (0.52 g . L^{-1}). Using *S. cerevisiae* TMB 3500 (Figure 10-B), xylose concentration remained constant until the end of fermentation because this yeast is not able to consume pentoses [53, 59]. Glucose was depleted after 20 hours and acetate started to be consumed at 25h fermentation, suggesting that hexose sugars were completely depleted at that time. Maximum ethanol concentration with *S. cerevisiae* TMB 3500 in bio-detoxified HSSL was 0.64 g . L^{-1} and was reached after 20 hours. Ethanol production was faster and more efficient than with *S. stipitis*. However, after this maximum content, ethanol was totally consumed due to aerobic condition and lack of other carbon sources.

An amount of cells corresponding to 1.08 x 10^8 CFUs . mL^{-1} was fixed as the required because this level is used in bioprocessing industries, generally leading to efficient fermentative processes. The results shown in Figure 11 ensure that the ratios obtained on the wet assays were correct, and the CFUs in the beginning (T_0) were very close to desired and required value. The number of *S. stipitis* cells was about three times higher in the end of fermentation (T_F), suggesting that sugars, organic acids and even ethanol consumption were used for biomass production and cell division. Aerobic conditions and abundance of available carbon sources favored growth and not ethanol production [61]. *S. cerevisiae* TMB 3500 CFUs were very stable during the fermentative process, indicating that the substrate was being used to maintain cell division levels. CFUs did not increase as in *S. stipitis* case, probably because carbon source was limiting, since TMB 3500 was not able to consume pentoses. The biomass evolution represented in Figures 10-A and 10-B is in agreement with the CFUs results, reinforcing these conclusions.

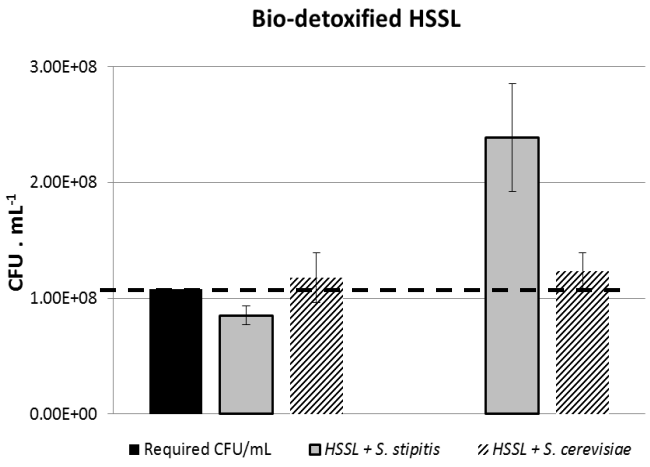


Figure 11 - *S. stipitis* and *S. cerevisiae* TMB 3500 CFUs at the beginning (T_0) and at the end (T_F) of fermentations using 100% bio-detoxified HSSL.

- 3.3.2.1. *S. stipitis* cell immobilization assays

Figure 12 shows the changes in the fermentation profile that immobilization of *S. stipitis* led to, as compared to the free-culture of the same yeast (Figure 10-A). Levels of xylitol, HMF and

furfural were very residual and, in some cases, were below the HPLC detection level. The variation of the pH of the bio-detoxified HSSL was very similar to the free-cell culture fermentation profile. It remains stable until the 25h and then increases slightly and continuously from 5.5 to 6.3 until T_F.

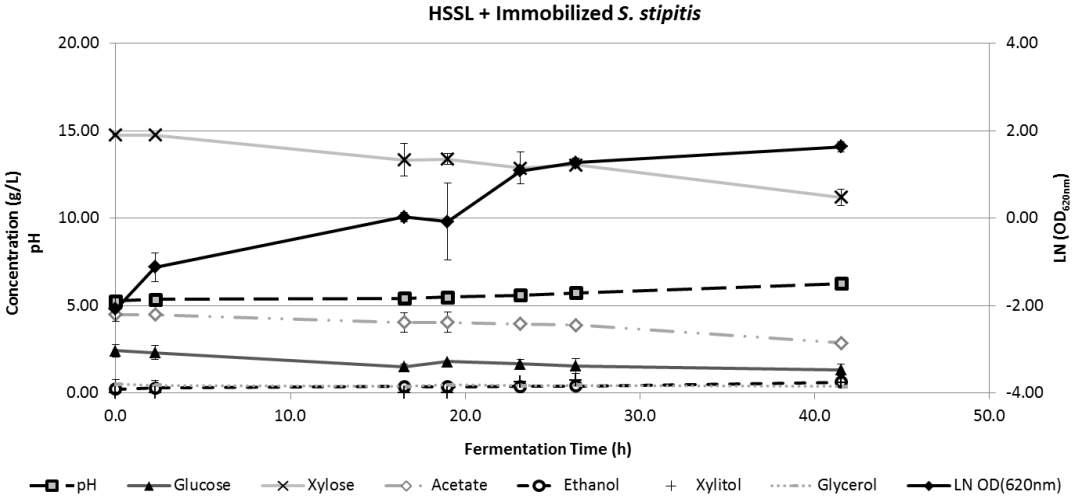


Figure 12 - Glucose, xylose, xylitol, glycerol, acetate, ethanol, HMF and furfural concentration profiles using immobilized *S. stipitis* in 100% bio-detoxified HSSL.

The consumption of glucose and xylose occurred at a lower but more stable and balanced rate, i.e. the substrates degradation occurred in the same way during all the process causing a sequential and not leveled increase on the fermentation products concentration (as observed in free-cell culture – Figure 10-A). The variations on xylose and acetate were bigger than in the suspended culture. However, glucose was not completely depleted, remaining in a residual level until the end of the fermentation. This result suggests that the calcium-alginate matrix may severely reduce the mass transfer of carbon sources and nutrients from the substrates over the layer when they are present in residual concentrations. In opposition to the results observed in *S. stipitis* suspended culture, the ethanol concentration continuously increased during fermentative process, reaching the maximum after 42h – 0.62 g . L^{-1}. The fact that ethanol concentration did not decrease when immobilizing yeasts suggests two possible behaviors and explanations:

- ethanol production during the time was much higher than in free-cell culture. The ethanol was continuously consumed during fermentation but the higher production compensated this effect and kept the variation positive. The protection given by the calcium-alginate matrix may have played an important role, since the cells were not so exposed to the inhibitors and hard conditions. Besides, this barrier limited the air transfer and might have optimized the oxygen content inside the bead, being the *S. stipitis* cells in perfect microaerophilic conditions. In addition, the disruption of beads that have occurred might have acted as an artificial and continuous

"donator" of fresh *S. stipitis* cells. These three factors are possible explanations for a higher ethanol production by immobilized cell;

or

- with immobilized cell culture there was not ethanol consumption during fermentation. The monomeric sugars were not depleted until the end of the fermentation and acetate consumption was steeper than in free-cell culture. This suggests that *S. stipitis* might have used other carbon sources instead of ethanol. The calcium-alginate matrix can contain traces of 60% bio-detoxified HSSL, which is a more suitable medium and *S. stipitis* might have used it as carbon source, avoiding the consumption of more complex substrates.

The *S. stipitis* biomass in 100% bio-detoxified HSSL continuously increased from the beginning until the 25h and at that time it started to stabilize (Figure 12). The entrapment process to immobilize made some cells to link to the outer layer of the matrix. The agitation and aeration levels applied on the bioreactors caused the release of the cells that were aggregated to the outside of the calcium-alginate matrix into the HSSL, increasing the CFUs in the liquid phase (bio-detoxified HSSL). Besides, during the fermentation some beads disruption occurred due to the shear stress. The agitation with a magnetic stirrer, the aeration system, the bioreactor geometry and the piping system (sampler, pH electrode, air exit, etc) are factors that may have contributed to the disruption of the cell beads since create mechanical and physical pressure on the matrix. In addition, sulphonate groups present in the HSSL are negatively charged in the medium and they strongly bind to positively charged groups that the calcium presents on the gel matrix. The addition of $CaSO_4$ to HSSL before the beginning of the fermentation aimed to avoid this binding, since there would be more Ca^{2+} available to bind to the sulphonate groups. With this addition the calcium-alginate matrix should remain stable until all Ca^{2+} from $CaSO_4$ would be depleted. The biomass curve evolution suggests that the results are in agreement with this prediction. However, disruption of some calcium-alginate barriers still occurred, releasing *S. stipitis* cells from beads to the medium.

- 3.2.2.2. Fermentation assays with pH control

Bio-detoxified HSSL and Domsjö hydrolysate are highly buffered media which means that pH should not vary unless significant changes or stress are applied. Since in the presented assays the pH increased after 25 hours on both the free-cell and immobilized fermentations, assays with pH control at 5.5 were performed in order to optimize the ethanol production. KOH 3M or H_2SO_4 3M were pumped by an automatic control system to fermentative processes in order to maintain the pH between 5.2 and 5.8 (optimal at 5.5).

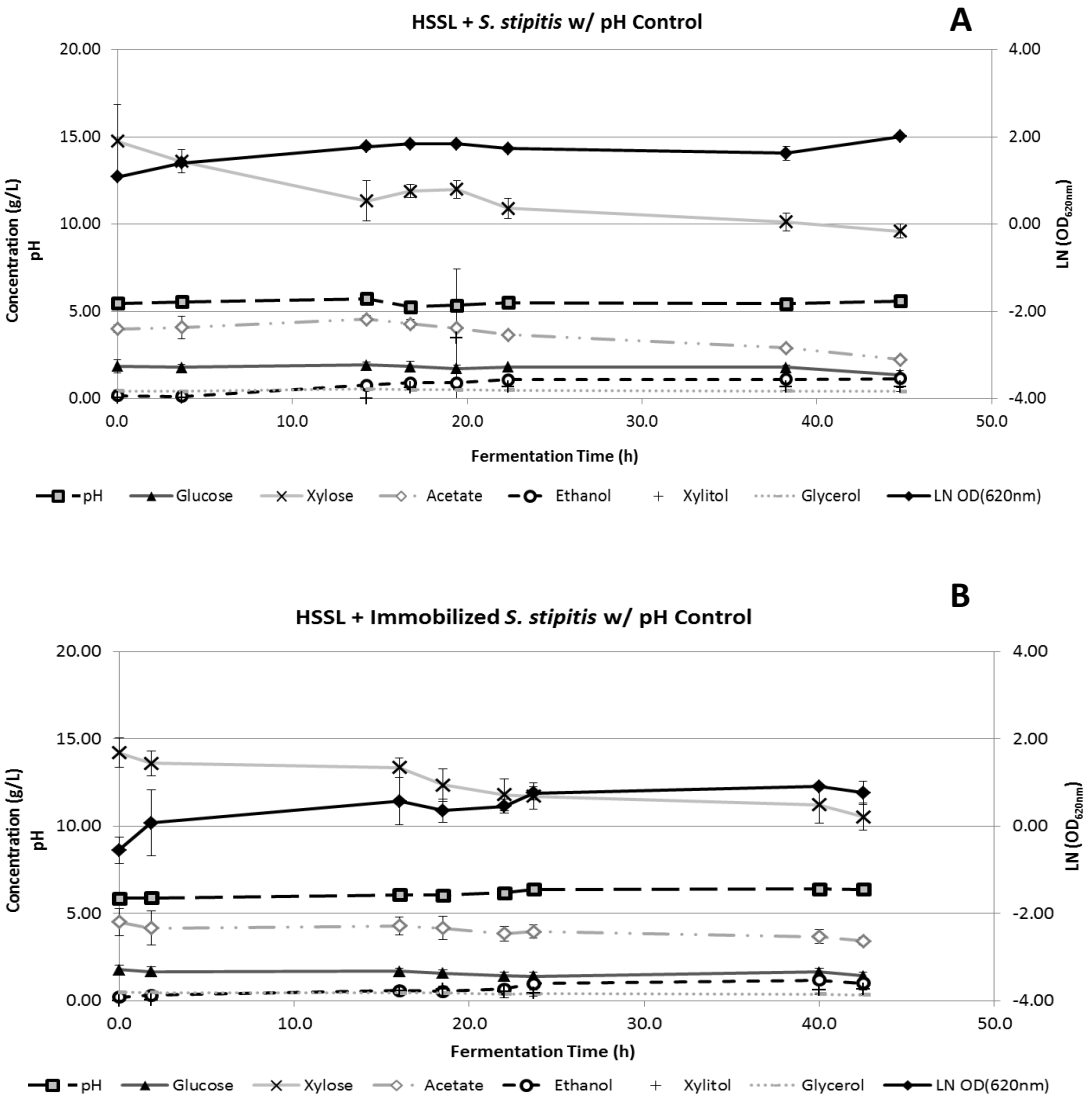


Figure 13 - Glucose, xylose, xylitol, glycerol, acetate, ethanol, HMF and furfural concentration profiles using free (13-A) and immobilized (13-B) *S. stipitis* in 100% bio-detoxified HSSL with pH control at 5.5.

Figure 13 presents the time courses for the batch tests performed by *S. stipitis* free-cell (Figure 13-A) and immobilized (Figure 13-B) cultures in 100% bio-detoxified HSSL with pH control at 5.5. In both cases, the maintenance of pH value did not change the profiles of biomass, xylitol, HMF and furfural.

Using free *S. stipitis*, the pH maintenance led to a more efficient consumption of xylose and acetate (Figure 13-A). More than 5 g . L^{-1} of xylose were consumed in 50h. Acetate started to be consumed after 18h and the uptake rate was slightly higher than that for the free-cell culture without pH control (Figure 10-A). However, the results suggested that the pH control might have a small negative effect on the glucose consumption since only 0.3 to 0.5 g . L^{-1} were metabolized, in opposition to 0.9 to 1.0 g . L^{-1} on free-cell culture without pH control. This decreased the

volumetric ethanol yield and productivity of the process but not significantly, since the amount of glucose on HSSL was residual compared to xylose and acetate. The maximum ethanol concentration was obtained at the end of fermentation (46h) – 1.07 g . L^{-1}. However, at 21h ethanol reached 0.73 g . L^{-1}, which is almost two times more that the value obtained with free culture with no pH control (0.43 g . L^{-1}). This result means that the accumulation of ethanol was more efficient and that the production occurred at a higher rate, leading to the achievement of better fermentative parameters.

The effects of the pH control on the substrates uptake and ethanol production in the fermentations with immobilized *S. stipitis* cells were very similar to those obtained with free-cell cultures, but in a smaller scale. Figure 13-B shows that the consumption of the carbon sources was similar to the immobilized assays without pH control (Figure 12). However, pH control led to an accumulation of a higher ethanol concentration. This suggests that the control of the initial pH at 5.5 is essential to optimize the fermentation efficiency, being the xylose and glucose more directly converted to ethanol. The maximum ethanol concentration was 1.18 g . L^{-1} and was reached after 40h, which constitutes an improvement of more than 2-fold in comparison to the process without pH control.

A synergistic effect of the maintenance of the pH at 5.5 and the cell immobilization was verified. The immobilization by itself had already had a significant positive effect on the fermentation efficiency, but the addition of the pH control improved the ethanol production. The results suggest that the variable "cell immobilization and pH control" is the most effective for the fermentation of 100% bio-detoxified HSSL into ethanol.

- **3.3.3. Domsjö hydrolysate Vs Bio-detoxified HSSL**

Table 9 summarizes ethanol production for the different tested conditions. The highest maximum ethanol yield (0.440 g ethanol . g $sugars^{-1}$) and productivity (0.885 g ethanol . L^{-1} . h^{-1}) were obtained with *S. cerevisiae* TMB 3500. These results confirm the SSSL potential to be bioprocessed into ethanol or another value-added bioproduct as well as the capacity of industrial *S. cerevisiae* strains to ferment it.

TMB 3500 had a slightly weaker performance in bio-detoxified HSSL, probably as the result of higher amount of pentoses and lower content of hexoses present in this SSL. However, it was the second highest ethanol yield obtained (0.36 ethanol . g $sugars^{-1}$).

Bioprocess using suspended *S. stipitis* without pH control led to the lowest maximum ethanol yield (0.09 ethanol . g $sugars^{-1}$) and productivity (0.010 g ethanol . L^{-1} . h^{-1}), that are about one-third of the same parameters obtained with TMB 3500 in the same substrate. However, low

ethanol production efficiency with *S. stipitis* was probably arising from the aerobic conditions, since this yeast needs microaerophilic conditions to produce ethanol in an efficient way [100, 101].

Table 9 – Maximum volumetric ethanol yields and productivities, substrate uptake rate and fermentation efficiency in terms of ethanol, obtained with *S. cerevisiae* TMB 3500 and *S. stipitis* in 100% Domsjö hydrolysate and 100% bio-detoxified HSSL with or without pH control.

		SSL	pH Control	$Y_{P/S}$ max	P_E Ymax	P_E 20-25h	r_S Ymax	Effic. (%)
***S. cerevisiae* TMB 3500**	**Free-culture**	Domsjö	No	**0.440** ± 0.02	**0.885** ± 0.16	**0.833** ± 0.01	**0.41** ± 0.04	**86.3** ± 0.0
		HSSL	No	**0.355** ± 0.06	**0.033** ± 0.00	**0.021** ± 0.01	**0.10** ± 0.01	**69.6** ± 0.1
S. stipitis	**Free-culture**	Domsjö	No	NA	NA	NA	NA	NA
		HSSL	No	**0.088** ± 0.02	**0.010** ± 0.00	**0.003** ± 0.00	**0.11** ± 0.00	**17.3** ± 0.0
			Yes	**0.140** ± 0.01	**0.034** ± 0.01	**0.020** ± 0.00	**0.15** ± 0.02	**27.5** ± 0.0
	Immobilized	HSSL	No	**0.111** ± 0.04	**0.006** ± 0.00	**0.008** ± 0.00	**0.09** ± 0.05	**21.8** ± 0.1
			Yes	**0.191** ± 0.06	**0.020** ± 0.00	**0.023** ± 0.00	**0.13** ± 0.03	**37.5** ± 0.1

NA → Not applicable
$Y_{P/S}$ max → Maximum ethanol yield (maximum g ethanol . (g glc + g xyl)$^{-1}$)
P_E Ymax → Volumetric ethanol productivity at the maximum yield (maximum g ethanol . (L . h)$^{-1}$)
P_E 20-25h → Ethanol productivity after 20-25h of fermentation (g ethanol . (L . h)$^{-1}$)
r_S Ymax → Substrate (glc + xyl + acetate) uptake rate at the maximum ethanol yield (g substrate . (L . h)$^{-1}$)
Effic. → Fermentation efficiency considering the maximum theoretical ethanol yield (0.51 . g ethanol . (g sugars)$^{-1}$)

The immobilization of *S. stipitis* cells led to the achievement of higher ethanol yields than with suspended cells. This reinforces the theory that the calcium-alginate matrix acted as a protection to inhibitors and hard conditions, limited air transfer thereby creating microaerophilic conditions and the disruption of beads might have acted as an artificial and continuous starter of fresh *S. stipitis* cells [131]. The volumetric ethanol yield using immobilized yeast increased 1.3-fold and 1.4-fold with and without pH control at 5.5, respectively. However, fermentations with immobilized cells are the unique cases that provided productivities in the middle term of the batch tests (20-25h) higher than at the time of the maximum $Y_{P/S}$. These results might be due to the mass

transfer limitations of substrates and products over the immobilization matrix, slowing the ethanol accumulation process on the medium.

The pH control led to improvements in the ethanol production with both suspended and immobilized *S. stipitis*. An 1.6-fold (suspended) and 1.7-fold (immobilized) increase in the maximum ethanol yields occurred with this yeast on 100% bio-detoxified HSSL. The pH control had a higher positive impact on the ethanol fermentation efficiency than the immobilization, meaning that pH 5.5 might be the optimal for HSSL fermentation by *S. stipitis* and immobilization process can still be improved. The lowest productivities ($P \leq 0.010$ g ethanol . L^{-1} . h^{-1}) were attained with suspended and immobilized *S. stipitis* in bio-detoxified HSSL without pH control. In contrary, the pH control in suspended *S. stipitis* led to the highest ethanol productivity of this yeast (0.034 g ethanol . L^{-1} . h^{-1}), suggesting the importance of this parameter to increase the ethanol production rate.

The substrate uptake rates at the maximum ethanol yield (r_S Ymax) were determined for each condition and the highest value was obtained when TMB 3500 performed the Domsjö hyd. fermentation. This value was at least 3 times higher than the r_S Ymax for the other conditions, which means that the substrate consumption was very pronounced in this case. In fact, the highest r_S Ymax was obtained in the shortest fermentation time period. This means that the substrate conversion occured at a high rate and most of the substrate was directed to the ethanol production route, resulting in the highest ethanol productivity. When HSSL was used, r_S Ymax were relatively similar, which suggests that this parameter varies with the experimental conditions (e.g. immobilization, pH control) and not with the microorganism used.

The *S. stipitis* immobilization led to lower r_S Ymax than for the same condition in *S. stipitis* suspended culture. This result reinforces the negative effect on the substrate uptake that the calcium-alginate matrix might have created. It also shows that the substrate was more efficiently used than in free-culture, since less carbon source consumed led to higher fermentation efficiencies.

pH control at 5.5 in *S. stipitis* fermentations led to an increase on the r_S Ymax and on the fermentation efficiency. This means that more substrate was used but also that ethanol production was favored.

Figure 14 compares the CFUs per mL obtained at the beginning (T_0) of the fermentation with the required (1.08×10^8 CFUs . mL^{-1}) and CFUs in the end of the process (T_F). The CFUs obtained at T_0 with free-cell cultures are very close to the previously stated as the required CFUs, except in the cases of *S. stipitis* without pH control (explained in section 3.2.2.) and *S. stipitis* with pH control. In the last one, the number of colonies in the beginning was too high compared to the

required CFUs and this fact might explain the high ethanol yield and productivity obtained. This CFUs level should be related with an error during the wet weight measure and/or transfer process.

S. cerevisiae CFUs are stable during the process since the value at T_F is very similar to the obtained at T_0, meaning that the substrates are being metabolized to maintain the growth rate and, simultaneously, to produce ethanol.

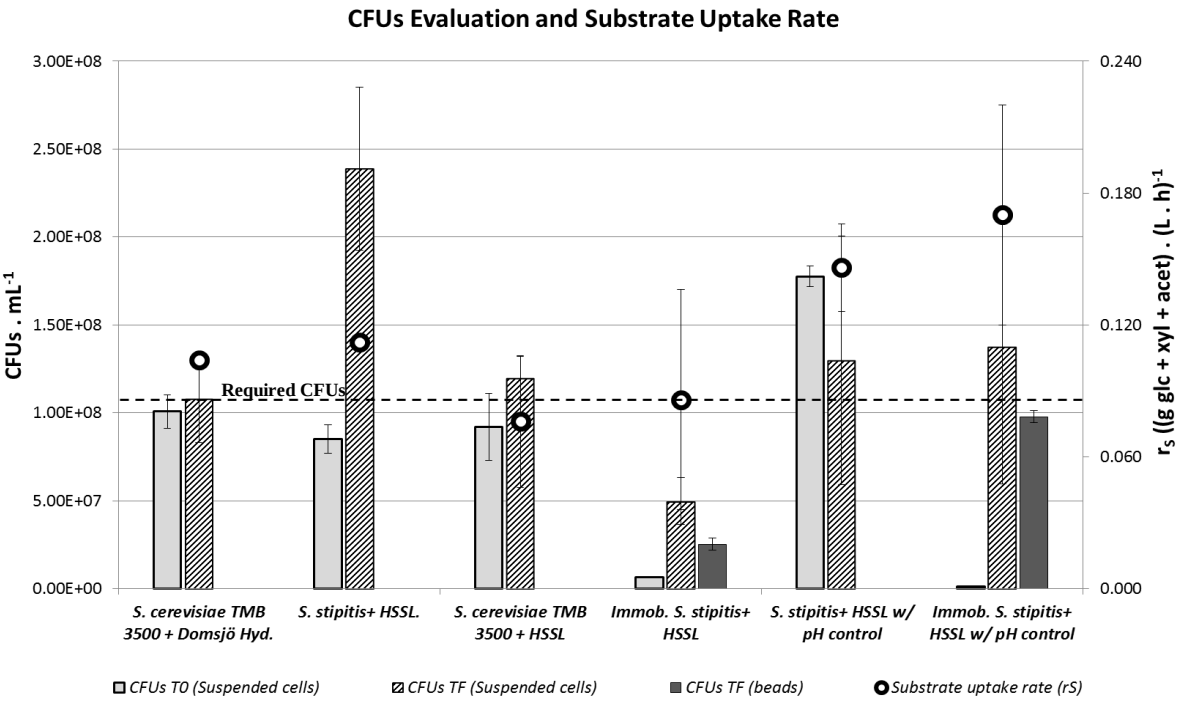


Figure 14 – CFUs per mL of SSL at the beginning (T_0) and at the end (T_F) of the different fermentations. In the case of immobilized cells, CFUs at T_F were determined from the suspended cells and by the disruption of the beads. The circles represent the substrate uptake rate of all process for each condition.

With respect to immobilized cells, the number of colonies in the medium at T_0 is around zero, since they should be inside the calcium-alginate matrix. Without pH control, CFUs from the medium increased up 7-fold which proves the beads disruption, that consequently led to the increase of viable biomass. The beads that remained until the end of the process were separated and disrupted in order to evaluate the CFUs and there was an increase of 4 times. When pH control was applied, the final colonies number were even bigger, having increased 130-fold and 93-fold for the suspended cells and cells that were inside of the beads, respectively. The total amount of CFUs in the end of fermentations performed by immobilized cells is the sum of the viable colonies that are suspended in the SSL and inside the beads. Considering this total amount, CFUs with immobilized *S. stipitis* increased up approximately 12-fold without pH control and more than 300-fold with pH control. These results showed the important positive effect that pH control at 5.5 and immobilization cause on the cell viability in SSLs. Both parameters led to considerable increases of the CFUs in the end of fermentation, resulting in better fermentation performances.

Substrate uptake rate of all fermentation processes (r_S) was determined for both yeasts and SSLs. It is possible to conclude that r_S is directly related to CFUs evolution. The highest r_S were obtained in the fermentations performed by *S. stipitis* with pH control. This control led to the highest evolution on the CFUs. On the other hand, TMB 3500 led to lower r_S and almost no variation occurred in the CFUs from T_0 to T_F. These results reinforce the previous conclusion that the substrate was more efficiently metabolized into ethanol by the TMB 3500, keeping the viable cells number stable. In general, ethanol production parameters were lower for *S. stipitis.* In these fermentations CFUs increased until the end. This suggests that most of the substrate was conducted for aerobic respiration and not for alcoholic fermentation, resulting in high biomass productivity but not in ethanol production.

When Domsjö hyd. was used as substrate for TMB 3500, r_S Ymax (0.41 ± 0.04) was about 4 times higher than r_S (0.08 ± 0.03). This suggests that the most efficient stage of the fermentation happened at the early beginning of the process. Thus, it is possible to conclude that this process started earlier and occurred faster than the other fermentations. In the other tested conditions, r_S Ymax values were similar to r_S obtained. This means that the yeast maintains approximately the same efficiency during the first 50 hours of fermentation.

During fermentation, the beads structure was affected by the chemical compounds, causing the disruption of the immobilization matrix and, consequently, releasing *S. stipitis* cells to medium. Figure 14 shows that the addition of $CaSO_4$ to avoid the beads disruption was partially successful since even with shear stress (mechanical and physico-chemical actions), there were beads that resisted during all the fermentative process. The fixation of the initial pH at 5.5 might have contributed to a better stability of the calcium-alginate matrix, since a higher number of cells were extracted from the calcium-alginate beads, showing that more beads resisted to the disruption during fermentation with pH control.

- **3.2.4. *S. stipitis* beads study/observation**

S. stipitis beads from the beginning and from the end of the fermentation were submitted to a microtome and microscope analysis in order to evaluate the cells distribution and the effect of HSSL on the calcium alginate-matrix. Immobilized *S. stipitis* from the assays with and without pH control were analyzed but there were no visible changes on the microscope. The results showed on Figures 15 and 16 correspond to the fermentations with control of the initial pH at 5.5.

In the beginning of the fermentation (Figure 15) the cells were widely spread inside the bead, meaning that the immobilization process occurred as predicted. The shape of the beads is approximately rounded, which should facilitate mass transfer. A perfect and uniform round shape and smaller beads size would maximize the efficiency of the transfers and avoid limitations that

might occur because of the physical barrier, but this could be only analyzed with more accurate technical equipment [132].

Figure 16 shows cells distribution in the end of fermentation process. It is possible to verify that in the end of fermentation there is a higher number of cells close to the calcium-alginate outer layer. This means that the cells have grown in the opposite direction of the cortex. The nutrients and carbon sources are more available in the area close to the outer layer, since the mass transfer limitations to the cortex are effective. The cells in the outer layer have more access to the substrates from the HSSL. On the other hand, it was expected that the *S. stipitis* cells would reproduce themselves mostly in the cortex area in order to be more protected from the inhibitory compounds.

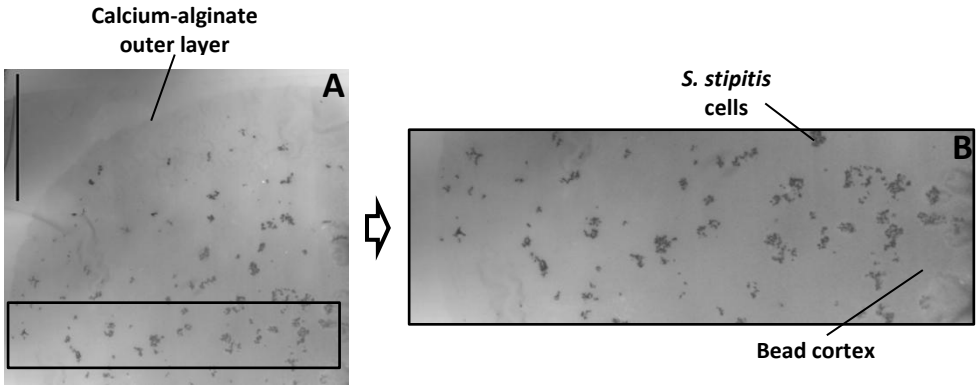


Figure 15 – Transverse section of a calcium-alginate bead with *S. stipitis* cells distribution at the beginning of the fermentation (T_0). Figure 15-A was taken on a optical microscope with 400x magnification and figure 15-B corresponds to a detailed image of the square present in 15-A.

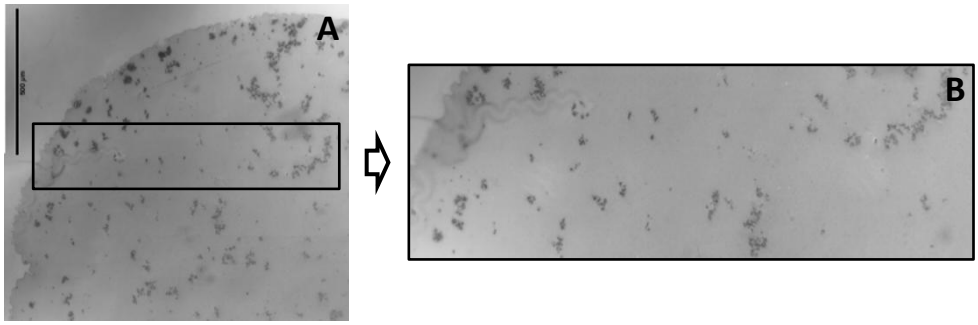


Figure 16 – Transverse section of a calcium-alginate bead with *S. stipitis* cells distribution at the end of the fermentation (T_F). Figure 16-A was taken on an optical microscope with 400x magnification and figure 16-B corresponds to a detailed image of the square present in 16-A.

The possibility of distinguish the viable cells amongst the total number cannot be achieved with the fixation and embedding processes utilized. An improvement in this field or the use of another technique should allow the analysis of the areas where viable yeast cells are located in the end of the fermentation.

4. Conclusions

The screening assays showed differences between *S. stipitis*, *S. cerevisiae TMB 3720* and *S. cerevisiae* TMB 3500 in six different percentages of two Spent Sulphite Liquors. Moreover, the three yeast strains behave similarly with 40% Domsjö hydrolysate or 60% bio-detoxified HSSL. These two percentages enabled a good compromise between the amount of SSL to bioprocess, the ability of the yeast to grow on SSL and the time that each microorganism took to reach stationary phase.

It was shown that methods and/or techniques used for the wet assays shall be optimized or changed for *S. cerevisiae* TMB 3720, since the results with this flocculating yeast were not as reproducible as for the other two yeast strains. In contrast, ratios of CFUs and wet weight on 40% SSSL and 60% HSSL were high, reproducible and similar for *S. stipitis* and *S. cerevisiae* TMB 3500. Thus, these were the two strains selected for fermentations.

The highest maximum ethanol yield and productivity were obtained in the fermentations using *S. cerevisiae TMB 3500* in 100% Domsjö hydrolysate, being these the conditions with more potential to an industrial application. Although *S. cerevisiae* cannot naturally metabolize pentoses, the most abundant sugar on HSSL, the fermentation on 100% bio-detoxified HSSL led to the second highest ethanol yield. Both results confirm the natural ability of *S. cerevisiae* strains of efficiently produce ethanol from lignocellulosic-based by-products.

Although *S. stipitis* provided the lowest ethanol productivity amongst the fermentations with free-cell cultures, an optimization on the supplied oxygen in the fermentor should lead to better ethanol fermentation efficiencies. *S. stipitis* is a microaerophilic strain an O_2 concentration is determinant for an efficient ethanol production.

Immobilization and pH control at 5.5 led to significant improvements in ethanol production from 100% bio-detoxified HSSL using *S. stipitis*. When applied simultaneously, these two factors increased the volumetric ethanol yield by 2.2-fold. Immobilization acted as a protection for the inhibitory compounds and pH control allowed the maintenance of the pH medium near to the optimal for ethanol production.

With the results of this research project, it was possible to compare the two most widely studied hexose-fermenting and pentose-fermenting yeasts and how they respond to the use of two different industrial by-products substrates. The effects of pH control and/or immobilization were also tested in the bio-detoxified HSSL fermentations with *S. stipitis*. This initial study has contributed to a better knowledge of the fermentative metabolism and physiological characteristics of *S. stipitis* and *S. cerevisiae*. A better understanding of mechanisms used in the fermentations by these yeasts is essential to optimize technologies and allow an efficient and sustainable large-scale production of second-generation bioethanol.

5. Future Work

According to the results of this project, the next stage should involve the the optimization of the microaerophilic conditions on *S. stipitis* fermentations to find and reach the optimal value of oxygen supply; the repetition of *S. stipitis* fermentations on Domsjö hydrolysate in order to verify the efficiency of this yeast on an undetoxified substrate; and, lastly, test *S. stipitis* in free and immobilized cell culture towards undetoxified HSSL.

6. References

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